A robust, efficient and flexible method for staining myelinated axons in blocks of brain tissue

By: Douglas Wahlsten, Frederick Colbourne, Richard Pleus

Wahlsten, D., Colbourne, F., and Pleus, R. (2003) A robust, efficient and flexible method for staining myelinated axons in blocks of brain tissue. *Journal of Neuroscience Methods*,123: 207-214.

Abstract:
Previous studies have demonstrated the utility of the gold chloride method for en bloc staining of a bisected brain in mice and rats. The present study explores several variations in the method, assesses its reliability, and extends the limits of its application. We conclude that the method is very efficient, highly robust, sufficiently accurate for most purposes, and adaptable to many morphometric measures. We obtained acceptable staining of commissures in every brain, despite a wide variety of fixation methods. One-half could be stained 24 h after the brain was extracted and the other half could be stained months later. When staining failed because of an exhausted solution, the brain could be stained successfully in fresh solution. Relatively small changes were found in the sizes of commissures several weeks after initial fixation or staining. A half brain stained to reveal the mid-sagittal section could then be sectioned coronally and stained again in either gold chloride for myelin or cresyl violet for Nissl substance. Uncertainty, arising from pixelation of digitized images was far less than errors arising from human judgments about the histological limits of major commissures. Useful data for morphometric analysis were obtained by scanning the surface of a gold chloride stained block of brain with an inexpensive flatbed scanner.
Keywords: Rat; Mouse; Corpus callosum; Throughput analysis; Reliability; Morphometry; Cerebral cortex; Measurement error

Article:

1. Introduction

Forebrain fiber tracts, especially the corpus callosum, are often subjected to morphometric analysis in a quest for sex and species differences (Driesen and Raz, 1995; Bishop and Wahlsten, 1997, 1999; Olivares et al., 2001) as well as effects of genetic mutations (Gonzales-Lima et al., 2001; Schmitt et al., 2001), steroid hormones (Fitch and Denenberg, 1998), prenatal ethanol (Livy and Elberger, 2001), traumatic brain injury (Bramlett and Dietrich, 2002), and environmental pollutants (U.S. Environmental Protection Agency, 1998). Schmued (1990) devised a gold chloride technique for myelinated axons in frozen sections mounted on glass slides, and Wahlsten and Schalomon (1994) adapted the method by staining a half brain en bloc in order to study large samples of mice in genetic investigations of commissure defects. In this paper, we extend the method by demonstrating its robustness and flexibility in assessing a wide range of morphometric traits. Because the efficiency in technician effort was of major interest, records of time for each step in the process were recorded and compared with another histological procedure.

2. Materials and methods

2.1. Animals

Adult (2-/7 months old) male Sprague-/Dawley and Wistar albino rats were obtained from the laboratory of F.C., and adult (3-/4 months) male and female BALB/ cWah1 mice were obtained from the breeding colony of D.W. All animals were housed and manipulated according to protocols approved by the Biological Sciences Animal Care Committee of the University of Alberta and guidelines established by the Canadian Council on Animal Care. Rats were housed singly in 41 × 22 × 18 cm plastic cages, while mice were group housed in 29 x 18 × 13 cm plastic cages. Both rats and mice were fed Purina 5001 rodent chow and Edmonton city tap water.

Rat cages contained Bed-o-cob bedding, while mice resided on Aspen-chip bedding. The colony rooms were on a 12 h: 12 h light—/dark cycle with lights on at 06:00 h.

2.2. Fixation

Rat brains were fixed in 10% neutral buffered formalin (Fisher) by one of two methods. (A) Intracardiac perfusion fixation was done after animals were anesthetized by an intraperitoneal injection of 80 mg/kg Somnotol (pentobarbital sodium) or inhalation of isoflurane gas (4% in a 7:3 nitrous oxide:oxygen mixture). The rat was then perfused with about 50 ml of physiological saline to remove vascular blood, followed by about 100 ml of fixative. Perfusion-fixed brains were removed from the skull after storage overnight in situ and then were placed in fresh fixative. (B) Immersion fixation was done by removing the brain quickly after either anesthesia with isoflurane or euthansia with carbon dioxide gas and then immersing it in 20 ml of fixative at room temperature. All mice were fixed by immersion in this study. Solutions used for fixation were all at room temperature (20 ± 1 ºC). Duration of fixation after perfusion or immersion for rats was 24 h, 48 h, or 7 days. Several perfusion-fixed rat brains were also stained after several months in the fixative. Time in fixative ranged widely for the mice.

2.3. Tissue preparation

Brain weight is an important measure in morphometric analysis because of the allometric relation of brain parts to the whole (Bishop and Wahlsten, 1999; Olivares et al., 2001). Although, whole brain size was not analysed in this study, details of the method we used are given because they were important for assessing the total time required. Each fixed brain was trimmed to a standard configuration by removing parts that were often damaged during extraction from the skull, including the olfactory bulbs, the paraflocculi of the cerebellum, the spinal cord caudal to the medulla oblongata, and portions of the optic and trigeminal nerves that protruded from the brain surface. The trimmed brain was gently blotted by rolling on a solid surface until excess fluid was removed from the surface, and then it was weighed to the nearest mg. After weighing, the brain was cut in half along the mid-sagittal plane with a razor blade while viewing from directly above with a stereo microscope. This simple and quick procedure yields satisfactory results when done carefully. The actual plane of section may depart from the true sagittal plane by a few degrees, but the error is usually inconsequential because cross-sectional areas of commissures are inversely proportional to the cosine of the angle between the actual and true planes. Even an error of 10º increases the area by only 2% (cosine of 10º is 0.985).

2.4. Staining

The staining solution is 0.2% gold chloride in phosphate buffer (1.8 g crystalline gold chloride, 0.33 g sodium phosphate monobasic monohydrate, 3.6 g sodium phosphate dibasic anhydrous, 9.0 g sodium chloride, 1000 ml distilled water). The fresh, unused solution can be stored at room temperature in a clear bottle for several months before use, even if the cap on the bottle is not absolutely air tight. We have not tested the stability of unused solution over a period of years. Once used for staining, the solution deteriorates rapidly, however. The gold chloride solution does not stain skin readily, in contrast with silver nitrate solutions, but our technicians routinely wear plastic gloves when handling brains stained in gold chloride solution. The powdered form of gold chloride is corrosive and needs to be handled carefully (http://fscimage.fishersci.com/mds/ 00703.htm), but once in a dilute solution it is not considered dangerous. The used solution is kept in a large jug for recycling or recovery of the gold and is not poured down the drain.

The half brain is removed from the fixative, blotted briefly, and then placed in 10 (mouse) to 20 ml (rat) gold chloride solution in a clear glass vial. The vial is placed in an oven at 37 ºC to accelerate the staining, but staining quality is good when done at room temperature. After about 40 min in the oven, rat brain commissures begin to appear as purple—brown structures. The experimenter decides when to stop the staining of each brain on the basis of its appearance. The goal is to obtain distinct commissures with a minimum of background staining. Invariably, if the commissures can be seen clearly with the unaided eye, the microscope image is entirely satisfactory for morphometry. In mice the staining requires more time, sometimes as much as 2 h, but the same visual criteria that apply for rat brain are satisfactory to obtain good results for mice. The species difference in time for staining probably reflects the greater density of myelinated axons in rat than mouse

commissures (Sturrock, 1980; Gravel et al., 1990). Once commissure staining is evident, the brain is removed from the gold chloride and promptly immersed in 10-20 ml of 2.5% sodium thiosulfate anhydrous (25 g in 1000 ml distilled water) for 5 min. It is then removed and stored in fresh 10% formalin, where it can remain in the dark for years with little apparent loss of stain intensity.

2.5. Extensions

Several other variations in the method were assessed. (A) It is known from previous work (Wahlsten and Schalomon, 1994) that more than one mouse brain can be stained in succession in the same gold chloride solution. Here we attempted to stain five rat brains in succession in the same vial of solution, the maximum number a technician could process sequentially in a standard work day. (B) In some of our tests, a brain failed to stain in a gold chloride solution that apparently had become exhausted. It was returned to 10% formalin for at least 1 week and then was stained in fresh gold chloride. (C) Half brains that had stained well to reveal commissures at the mid-sagittal plane were then cut in the coronal plane with a razor blade and stained again in gold chloride. (D) A well-stained half brain was sectioned serially at 40 microns in the coronal plane with a cryostat. Sections were mounted on glass slides and stained with cresyl violet to reveal Nissl substance.

2.6. Morphometry

The wet, stained half brain was attached to a glass cover slip by surface tension and then inverted under a microscope to obtain an image of the commissures at the mid-sagittal plane or a variety of structures in the coronal plane. More than one image was sometimes required to fit all parts of a large brain. Gray scale (8 bit) images were captured by a frame grabber at 640 × 480 pixels from a Sony SPT-M324 TV camera mounted on a Wild M3Z stereoscope using the GLOBAL LAB Image/2 program (Data Translation Inc.) running on a PC with Windows NT. The stored bitmap images were then measured with the ImageJ software from NIH (http://rsb.info.nih.gov/ij/index.html). Maximum lengths and cross-sectional areas were determined for the anterior commissure, hippocampal commissure, and corpus callosum. The anterior commissure included both the anterior and posterior parts as well as the commissural part of the stria terminalis. The corpus callosum did not include adjacent structures such as the dorsal commissure of the fornix, the longitudinal striae of Lancisius, or the superior fornix (Wahlsten and Schalomon, 1994). It was sometimes necessary to view the stained brain with the stereoscope in order to perceive fine distinctions that were not obvious in the stored image. In a few cases, an entire half rat brain was also scanned with a flatbed scanner (HP 4470c) at 2400 dpi to obtain a JPEG image.

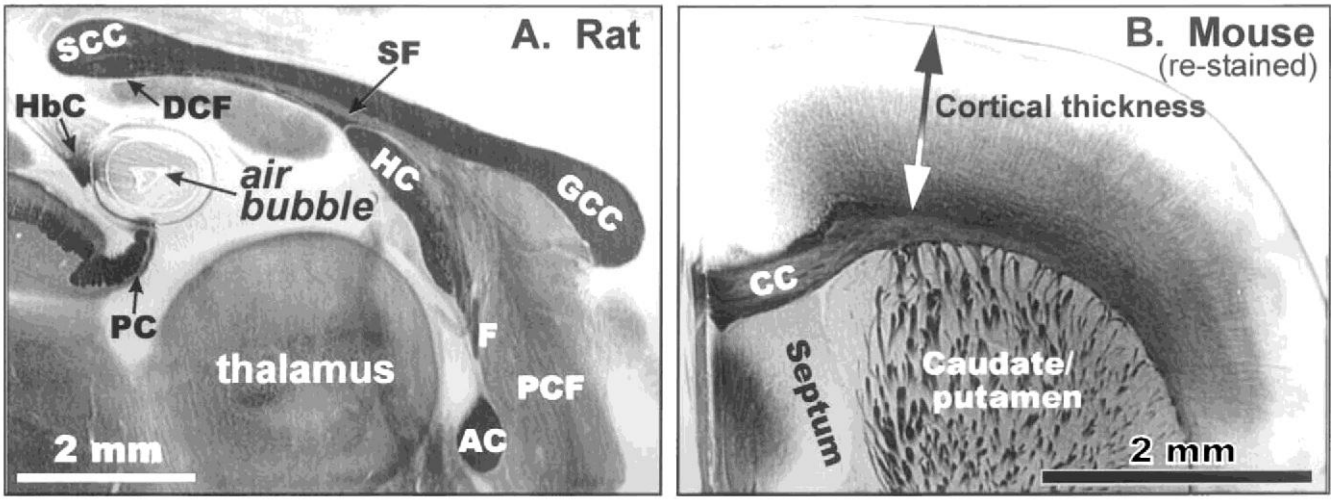

Fig. 1. (A) Sprague–Dawley rat brain fixed by perfusion, then bisected and stained with gold chloride after 1 month in formalin. Image was digitized as an 8-bit grayscale bitmap at 640 × 480 pixels, then enhanced to give higher contrast. All commissures appear as darkly stained structures, whereas more diffuse tracts such as the precommissural fornix (PCF) stain more lightly while showing distinct fibers. The air bubble was trapped in the third ventricle when the wet half brain was pressed against a glass coverslip for viewing. (B) BALB/cWah1 mouse brain that was first bisected and stained with gold chloride to reveal commissures, and then was cut as a slab in the coronal plane at the level of the anterior commissure and stained with gold chloride again. Small diameter myelinated axons are visible in the cerebral cortex, and the dorsal edge of the corpus callosum can be used as a reference point for measuring cortical thickness. Abbreviations: AC, anterior commissure; CC, corpus callosum; DCF, dorsal commissure of the fornix; F, columns of the fornix; GCC, genu of the corpus callosum; HC, hippocampal commissure; HbC, habenular commissure; PC, posterior commissure; PCF, precommisural fornix; SCC, splenium of the corpus callosum; SF, superior fornix.

2.7. Reliability and stability

Five weeks after commissure measurements were completed on 24 rat half brains, the same half brains were measured again by repeating the entire process of mounting the brain in the stereoscope, capturing an image, and measuring areas. This was done in a blind manner by the same experimenter who collected the first measurements. For four brains where one-half had already been stained, the other half was stained with gold chloride after an additional week in fixative.

2.8. Paraffin embedding and staining

Conventional morphometry is sometimes done by embedding the coronal brain slab in paraffin and staining serial sections with hematoxylin and eosin or some other general purpose stain. Dehydration in alcohol severely shrinks soft tissue (Rosen et al., 1990; Wahlsten and Bulman-Fleming, 1994), and processing in toluene or xylene dissolves lipids, a principal component of myelin, which distorts commissures. Nevertheless, in order to compare times required for different methods, two rat half brains were processed by the Biological Sciences Microscopy Service at the University of Alberta with this method.

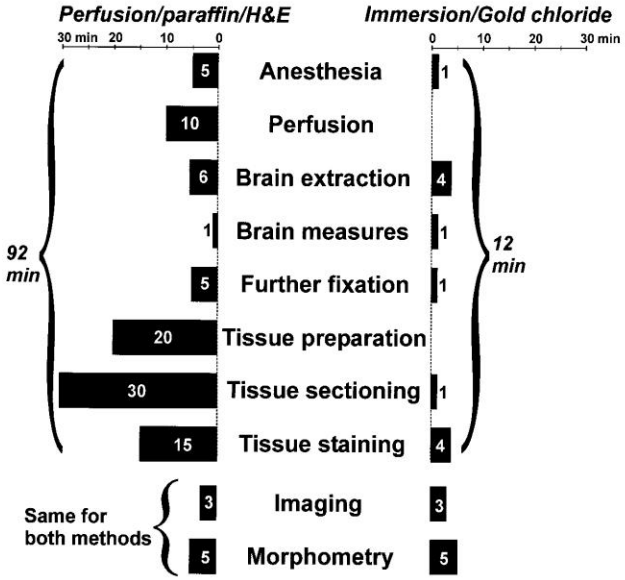

Fig. 2. Times in min required for each step in the process with two methods. The approximate total technician time per brain with the perfusion/paraffin/H&E serial section method was 92 min, versus 12 min for immersion fixation and then gold chloride staining of a bisected brain. For the gold chloride method, it was possible to extract a brain from the skull while the next animal was being anesthetized, but the death from carbon dioxide required more than 1 min. Staining a rat brain with gold chloride typically required about 40 min at 37 °C, but at least 10 brains could be stained and fixed at the same time. Time for imaging was based on image capture with a TV camera on a stereoscope, as described in the text.

3. Results

Examples of sagittal and coronal sections from rat and mouse brains stained en bloc are shown in Fig. 1. The resolution of smaller diameter myelinated axons is not as good as in 25—/40 micron sections on microscope slides because the stain penetrates the tissue block more than 25 microns, resulting in overlap of the finer fibers. Otherwise, the images obtained by en bloc staining are entirely satisfactory for morphometric measures. All variations in fixation methods yielded acceptable images, including both perfusion and immersion fixation as well as fixation ranging from 24 h to 7 months before staining. Taking immersion fixation and 24 h in fixative before staining as the fastest method, the gold chloride method requires about one-seventh the technician time that is needed for perfusion fixation followed by paraffin embedding, serial sectioning and staining in H&E (Fig. 2).

The test—/retest reliability of most measures was quite high, especially for the corpus callosum (Fig. 3). The standard error of estimate when the second measurement of the same structure is predicted from the first measurement gives a good indication of the size of measurement error in the same units as the measurement itself. For the observer, deciding which points determine the maximum length of the corpus callosum is an easy matter, and the two measurements taken more than a week apart differed on average (absolute values) by only 0.06 mm in a structure averaging 7.2 mm length, a difference of about 1%. The standard error was also small (0.07 mm). Measuring area requires the observer to choose a long perimeter that separates stained and unstained

tissue as well as the corpus callosum proper from adjacent structures, with the consequent greater opportunity for changing one's mind. Nevertheless, reliability of corpus callosum area was also quite high, considering the relatively narrow range of values in this sample of rats. The average absolute change between the two measures of corpus callosum area (0.13 mm^2) was about 4% of its average size. Reliability for the anterior commissure was slightly lower, which is consistent with the much smaller size of the structure that was digitized at the same magnification as the corpus callosum. Reliability for area of the hippocampal commissure was considerably lower, and it was significantly lower than the reliability (r = 0.91) for the anterior commissure (Fisher's Z transformed correlations, t = 2.4, P = 0.011). We believe this lower reliability resulted from differing judgments of the boundary at the anterior edge of the hippocampal commissure, an edge that is highly convoluted. The observer must also decide where the hippocampal commissure ends and the dorsal commissure of the fornix begins, and this discrimination is difficult for some brains. Nevertheless, the correlation between the two measurements of the hippocampal commissure was highly significant (r = 0.66, t = 4.12, df = 22, P = 0.0002).

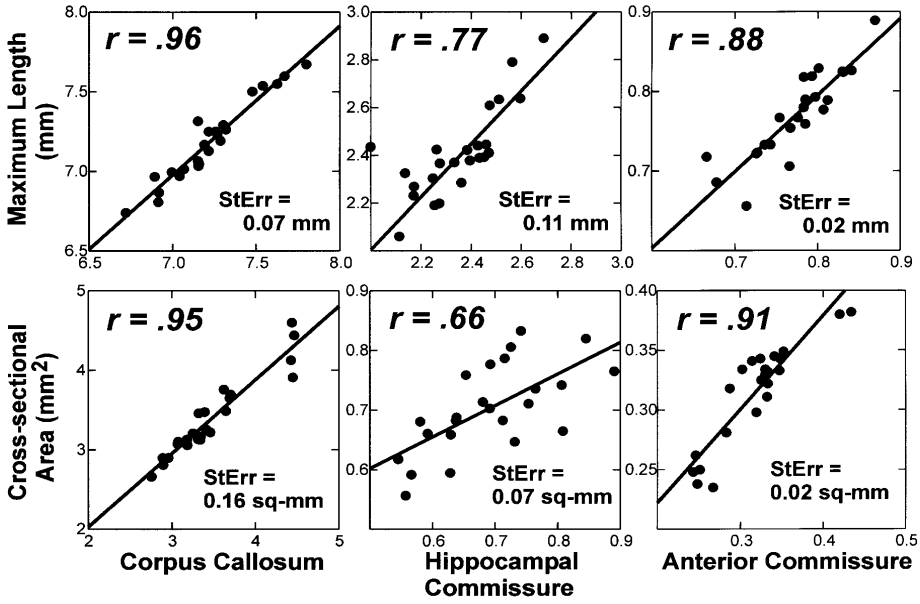

Fig. 3. Test–retest reliability, indicated by Pearson correlations (r) between measures on 24 bisected rat brains stained with gold chloride and the same half brains re-measured 5 weeks later. The standard error of estimate (StErr) from the linear regression equation is the standard deviation of the points around the straight line of best fit that predicts the second measurement from the first, which provides a good index of the degree of measurement error resulting from human judgments about the boundaries of commissures.

Stability of measurements was very good. For the 24 half brains re-measured after about 35 days, the average corpus callosum length increased by only 0.4% and its average area increased by 2%. Dimensions of the corpus callosum changed very little with an additional week in formalin before staining; for a sample of four rat brains, corpus callosum length increased 0.7% and area de-creased 3.1%, values well within the range of measurement error.

Video images were taken at a magnification that included all three commissures in most cases, which meant that the corpus callosum, being the largest of the three, was generally segmented into about 600 pixels along its length. Scanning the entire half rat brain with a flatbed scanner at 2400 dpi segmented the corpus callosum into about 650 pixels and provided an image that was adequate for most morphometric measures. As indicated in Fig. 4, however, the scanned image did not appear to be as sharp, possibly because of differences in focus between the stereoscope and scanner. More expensive scanners may give better results. The pixel resolution of both the TV camera and the scanner appeared to be adequate for morphometry because it was finer than the typical measurement error of 1-/4% attributable to differences in judgments by the same observer.

When five rat brains were stained in succession in one vial of solution in a single work day, all five gave acceptable results, and there was no obvious diminution in the quality of the stain. Leaving a previously used gold chloride solution at room temperature overnight or longer, however, generally resulted in failure to stain additional brains, even if only one brain had been stained in the solution. The chemistry of the gold chloride

reaction with neural tissue is very complex and mysterious, as occurs for several other heavy metal salts. Fortunately, we found that if a brain failed to stain in an evidently exhausted solution of gold chloride, it could be successfully restained a week later in fresh solution. Despite the many variations of the gold chloride method that we tested, in no case did we fail to stain a brain adequately. The quality of the staining, especially the amount of non-specific background staining, differed greatly among individual rat and mouse brains subjected to the same procedure, but the back-ground staining generally did not obscure the commissures. Differences among individual rats subjected to the same procedure far exceeded the minor differences between variants of the gold chloride procedure that we evaluated.

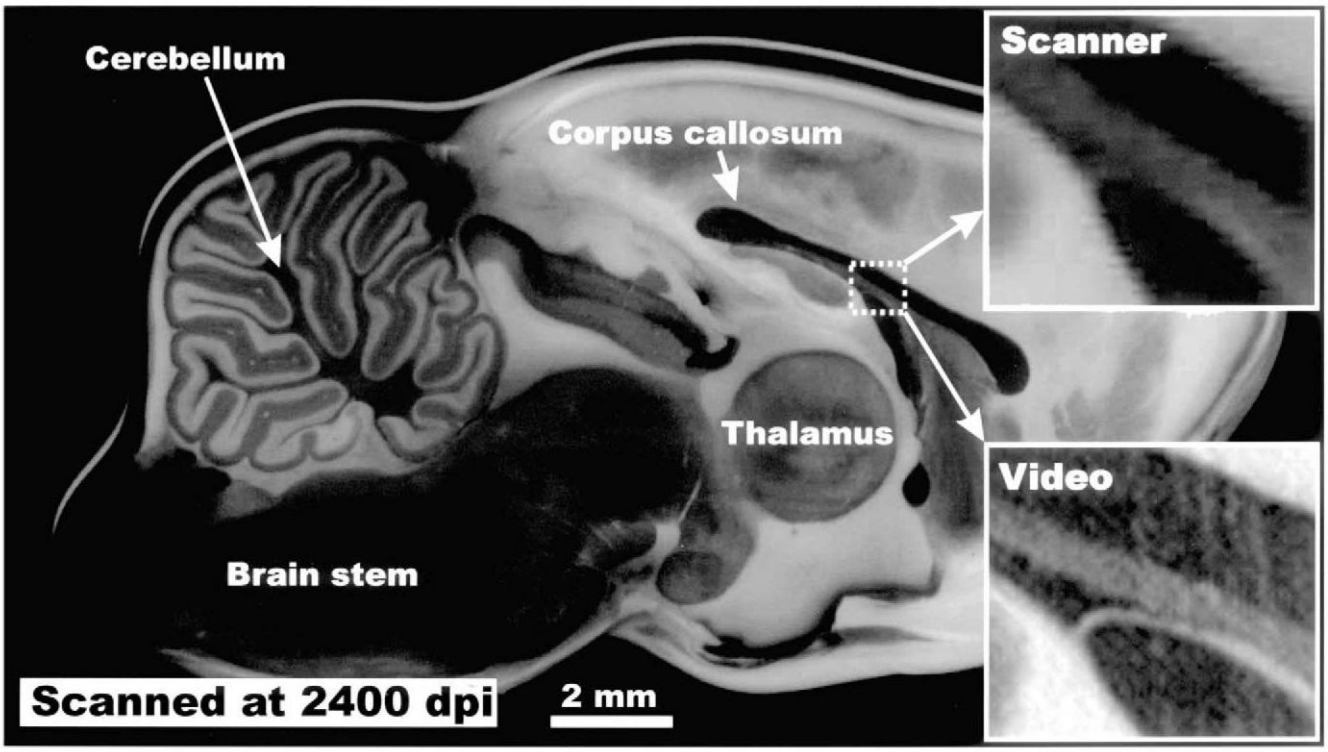

Fig. 4. Image of the same rat brain shown in Fig. 1A that was later taken in the JPEG format on a flatbed scanner at an interpolated 2400 dpi. Insets show finer details of the images from the scanner and the 640 × 480 pixel video image. The image obtained with a TV camera mounted on a stereoscope is obviously superior, despite the nominally equal number of pixels in the same areas.

After a half brain had been stained to reveal commissures at the mid-sagittal plane, it was then possible to cut coronal slabs in that half and stain again to reveal myelinated fiber tracts in a coronal section (Fig. 1B). The reason for this fortunate circumstance is that the gold chloride staining of the half brain penetrated less than 0.1 mm into the surface of the brain (Fig. 5). It was also possible to obtain excellent coronal sections on a cryostat from a previously stained half brain, then stain for Nissl substance with cresyl violet (Fig. 5).

4. Discussion

Previous studies have demonstrated the utility of the gold chloride method for en bloc staining of a bisected brain in mice (Wahlsten and Schalomon, 1994; Bishop and Wahlsten, 1999) and rats (Bishop and Wahlsten, 1999; Livy and Elberger, 2001). The present study shows that the method is approximately seven times more efficient with regard to technician time than a conventional approach involving perfusion fixation, paraffin embedding, and serial sectioning. The method is highly robust. We obtained acceptable staining of commissures in every brain, despite a wide variety of fixation methods, and a brain could be stained at a later date even if the first attempt failed because of exhausted staining solution. Relatively small changes were found in the sizes of commissures stored for several weeks after initial fixation or staining. A half brain stained to reveal commissures in the mid-sagittal section could then be sectioned coronally and stained again in either gold chloride for myelin or cresyl violet for Nissl substance. Uncertainty, about the limits of a myelinated structure that arose from pixelation of digitized images was far less than errors arising from human judgments about the limits of major commissures.

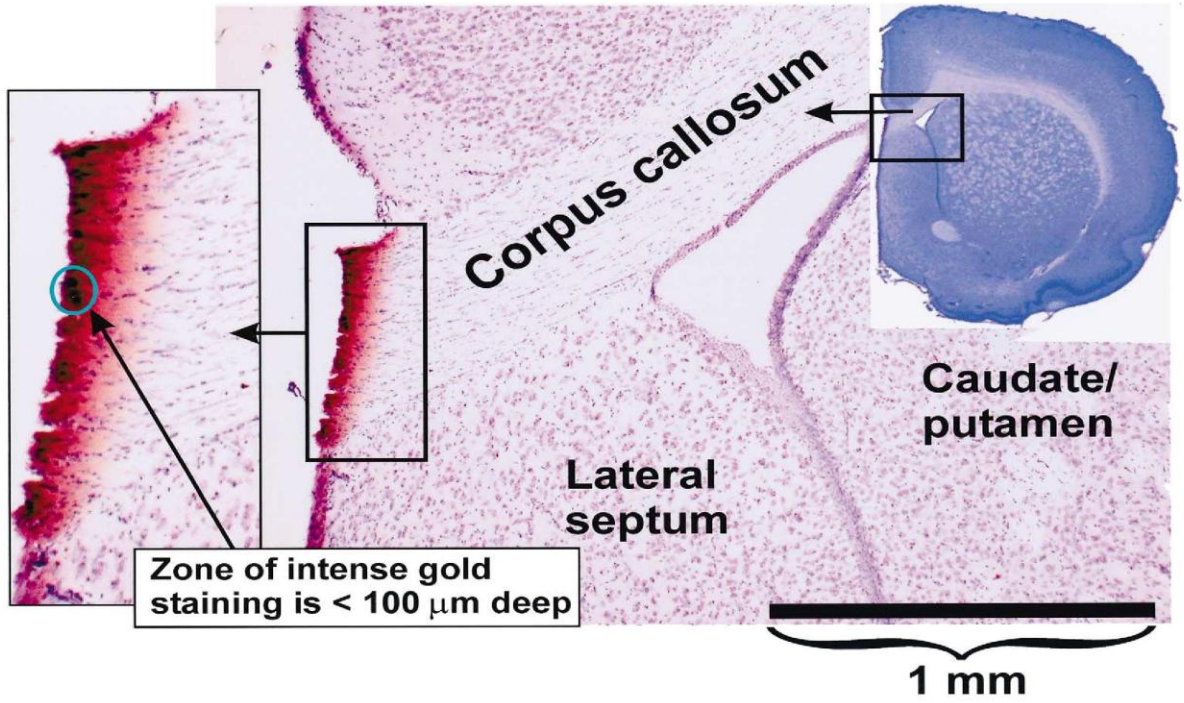

Fig. 5. Coronal section of a rat brain cut at 40 μm on a cryostat and then stained with cresyl violet to reveal Nissl substance, after the half brain was first stained with gold chloride for myelin. The quality of cresyl violet staining is excellent. As shown in the inset, the dark purple–brown gold chloride staining penetrated less than 0.1 mm into the brain.

Efficiency attainable with the gold chloride method applied to blocks of tissue is particularly beneficial for studies that require large samples of rats or mice. The least labor was involved when brains were fixed by immersion in formalin and stained only 24 h after they were extracted. Further efficiency was achieved by placing the stained block on a flatbed scanner to digitize the image. Thus, the method can yield good results without the use of intracardiac perfusion, a microtome or even a microscope, and it could easily be applied in field studies where limited facilities are available.

We do not argue that the en bloc gold chloride method will achieve higher quality anatomical data than all other methods. Microtome sections and higher power microscopy reveal more about the fine details of myelinated axons and intricate shapes and components of the commissures. By staining alternate coronal sections throughout the brain for myelin and Nissl substance, high quality morphometry is feasible. Provided enough (more than 10) sections are measured, cross-sectional areas of large commissures can be estimated by summing commissure thickness in a series of coronal sections (Rosen et al., 1990), and cortical thickness can be measured at specific anterior—posterior levels with respect to major landmarks. This approach is very labor intensive, however. Furthermore, microtome sections mounted on glass slides entail risks of distorting the tissue during mounting onto the slide, a problem that is minimized with en bloc staining.

Our findings suggest a procedure for obtaining excellent morphometric measures from the same brain in all three cardinal planes. First obtain a good image of commissures and cerebellar foliation at the mid-sagittal plane using gold chloride staining on a half brain, then obtain measures of cortical thickness and other features in coronal sections cut at desired anterior—/posterior levels of the brain, as seen in the sagittal section, and stained with either gold chloride or cresyl violet. The other half of the brain could be sectioned in the horizontal plane to study sizes of zones in the hippocampal formation. Relying on a unilateral measure of a bilaterally symmetrical structure adds the contribution of fluctuating asymmetry to measurement error, but for most areas of the brain this source of error is about the same size as the error incurred in measuring the same stained section twice.

References

Bishop K, Wahlsten D. Sex differences in the human corpus callosum: myth or reality? Neurosci Biobehav Rev 1997;21:581-/601.

Bishop KM, Wahlsten D. Sex and species differences in mouse and rat forebrain commissures depend on the method of adjusting for brain size. Brain Res 1999; 815: 358-66.
Bramlett HM, Dietrich WD. Quantitative structural changes in white and gray matter 1 year following traumatic brain injury in rats. Acta Neuropath 2002; 103:607-14.
Driesen NR, Raz N. Sex-, age-, and handedness-related differences in human corpus callosum observed in vivo. Psychobiology 1995; 23: 240-/7.
Fitch RH, Denenberg VH. A role for ovarian hormones in the sexual differentiation of the brain. Behav Brain Sci 1998;21:311-52.
Gonzales-Lima F, Berndt JD, Valla JE, Games D, Reiman EM. Reduced corpus callosum, fornix and hippocampus in PDAPP transgenic mouse model of Alzheimer's disease. Neuroreport 2001; 12: 2375-9.
Gravel C, Sasseville R, Hawkes R. Maturation of the corpus callosum of the rat: II. Influence of thyroid hormones on the number and maturation of axons. J Comp Neurol 1990; 291: 147-61.
Livy DJ, Elberger AJ. Effect of prenatal alcohol exposure on midsagittal commissure size in rats. Teratology 2001;63:15-22.
Olivares R, Montiel J, Aboitiz F. Species differences and similarities in the fine structure of the mammalian corpus callosum. Brain Behav Evol 2001;57:98-105.
Rosen GD, Sherman GF, Emsbo K, Mehler C, Galaburda AM. The midsagittal area of the corpus callosum and total neocortical volume differ in three inbred strains of mice. Exp Neurol 1990;107:271-6.
Schmitt JE, Eliez S, Warsofsky IS, Bellugi U, Reiss AL. Corpus callosum morphology of Williams syndrome: relation to genetics and behavior. Dev Med Child Neurol 2001;43:155-9.
Schmued LC. A rapid, sensitive histochemical stain for myelin in frozen brain sections. J Histochem Cytochem 1990;38:717-20.
Sturrock RR. Myelination of the mouse corpus callosum. Neuropath Appl Neurobiol 1980;6:415-20.
U.S. Environmental Protection Agency, 1998. Health effects test guidelines. OPPTS 870.63000: developmental neurotoxicity study. Washington, DC: Office of Prevention, Pesticides and Toxic Substances; report no. EPA 712-C-98-239.
Wahlsten D, Schalomon PM. A new hybrid mouse model for agenesis of the corpus callosum. Behav Brain Res 1994;64:111-7.
Wahlsten D, Bulman-Fleming B. Retarded growth of the medial septum: a major gene effect in acallosal mice. Dev Brain Res 1994;77:203-14.