Dark-field microscopy visualization of unstained axonal pathways using oil of wintergreen

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Abstract

Despite enormous progress in the development of new morphological techniques, there is still not a simple technique for visualization of the fiber architecture in the mammalian brain. To develop such a technique, thick (400–600 μm) sections of the rat, mice, calf or postmortem human brain were fixed in paraformaldehyde, dehydrated in a series of ethanol and finally immersed in methyl salicylate. The major principle of this newly developed method was to make the neural tissue transparent, and then utilize the ability of neuronal fibers to deflect and deviate light directed from the side to render them visible. Dark-field illumination was used to create illuminating rays of light arriving at an angle exceeding the collecting angle of the objective lens, thus causing only the axonal pathways to be visible as a bright silver silhouette against a dark background. As a result, a three-dimensional structure of the whole white matter of the brain slice became clearly viewable. This technique worked equally well for mammalian brain frontal, sagittal and horizontal sections, as well as for the spinal cord sections. The method was appropriate for verification of axonal fiber courses in brain slice preparations used in electrophysiological experiments, including special applications, such as visualization of axonal bundles within neural transplants. Due to its simplicity, the technique can be successfully used even in an amateur laboratory having basic microscopy equipment and reagents. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Despite enormous progress in the development of new techniques for studying nerve pathways in the CNS of mammals made in the last decades, few methods are available for visualization of the whole fiber architecture in the brain. The most known methods are staining with iron–haematoxylin (Weil et al., 1928; Anderson, 1929), Luxol Fast Blue (Kluver et al., 1953), Sudan-Black (Olson et al., 1990), and Black-Gold (Schmued et al., 1999). Another popular technique for fiber visualization is silver staining (Bodian, 1937; Davenport, 1929; Glei, 1946; Nauta et al., 1950; for review see Beltramino et al., 1993). In the last decades, methodological research primarily focused on tracing single axonal pathways with markers moving by axonal transport (Kristensson et al., 1971; Gerfen et al., 1984; Katz et al., 1984; Schmued et al., 1986) or with lipid soluble dyes drifting within the cell membrane (Honig et al., 1986).

While these and other techniques proved to be beneficial, they have certain limitations. Firstly, most are rather sophisticated, labour intensive, time consuming and many require expensive reagents. Secondly, the absolute majority of known tract tracing techniques deal with relatively thin (less than 100 μm) sections of neural tissue. A major drawback of thin sections is that they are virtually two-dimensional, and many sections must be pooled together for three-dimensional reconstruction of large segments of the fiber system in the CNS.

This report presented a different approach developed after years of experimentation to find a simple tech-
nique by which the whole fiber architecture of a thick unstained brain slice could be made viewable. The major principle of this method is to make neural tissue transparent, and then visualize axonal bundles without any staining, solely using their ability to deflect and deviate light rays directed at an angle which exceeded the collecting angle of the objective lens.

2. Methods

The method was originally developed using brains of calf (purchased in a local food store) and male adult Wistar rats (Institute for Experimental Medicine, Russia), as well as postmortem human brain samples (1st Medical Institute, St. Petersburg, Russia). Later its usefulness was also verified on adult female and neonatal (16 days) Wistar rats (Charles-River, Canada) and adult C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine).

The present experiments also employed fixed tissue pre-used in electrophysiological or morphological experiments as described elsewhere (Senatorov et al., 1993, 1995; Kolaj et al., 1998). During electrophysiological experiments, 400 or 500 μm slices were maintained for up to 8 h at room temperature in oxygenated artificial cerebrospinal fluid, and then fixed by immersion in 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4 (PPB), at room temperature. Some experiments utilized ‘leftover’ brain tissue from animals used in morphological experiments (Senatorov et al., 1995), in which rats or mice were over-anaethetized with sodium pentobarbital and perfused transcardially with 100 ml of saline followed by perfusion with 200–300 ml PPB and then postfixed by immersion in PPB (Senatorov et al., 1993). Calf and postmortem human brain tissues were fixed by immersion in 10% formalin. Following fixation, 200–600 μm thick sections were cut with a set of parallel blades. Alternatively, and upon availability, a chopper or a device with vibrating blade (e.g. vibratome or vibrating blade microtome) were used. In all experiments, fixed sections were dehydrated by ascending series of ethanol, 70, 95 and 100%, 10 min each, and left in a second change of 100% alcohol for 1–2 h. Finally, sections were immersed in methyl salicylate (2-hydroxybenzoic acid methyl ester, also known as oil of wintergreen) for about 10–20 min, till they became visually transparent.

For observance under a microscope, slices were mounted in methyl salicylate on glass slides and coverslipped. Fiber architecture was best observed using a low-power objective, e.g. 2×, 5× or 10× with a numerical aperture lower than the numerical aperture of the dark-field condenser.

3. Results

As the result of methyl salicylate exposure, lipids and some other organic molecules were removed from the brain slices, which made them transparent and practically ‘invisible’ under normal illumination. However, when slices were illuminated by a hollow cone of light striking from the dark-field condenser at an angle exceeding the collecting angle of the objective lens, axonal bundles became clearly visible as a bright silver-white substance against a dark background. In the experiments, frontal, sagittal and horizontal sections of the brain were successfully used without any difference in results. In CNS regions containing large axonal bundles running within a surrounding mass of gray matter such as the forebrain, the three-dimensional course of axonal bundles can be clearly viewable due to gray matter transparency (Fig. 1A). If slices were immersed in ethanol without pre-fixation in paraformaldehyde, the preparation had to be viewed immediately after a very short (few minutes) incubation in methyl salicylate. Otherwise, the portion of thinner fiber bundles quickly became darker and less visible. The brain slices prefixed with paraformaldehyde required longer methyl salicylate immersion (as described in Section 2) and can be kept there for days or even weeks.

This dark-field microscopy technique was successfully employed for verification of axonal pathways in slice preparations previously used in electrophysiological experiments, e.g. cortico–striatal and thalamic slices, and brain slices containing embryonic transplants. For example, Fig. 1B shows a photo of a horizontal slice through the rat thalamus. This slice was used in an electrophysiological experiment to study the thalamo–cortical connection (unpublished material). In this experiment, the technique was employed to choose the best angle to cut a 400 μm horizontal brain slice, which contained axons running all the way through the auditory thalamo–cortical pathway, and to visualize the course of fibers between stimulating and recording electrodes.

In addition to brain tissue, spinal cord slices were also used, and it was found that the technique worked equally well (Fig. 1C). Methodologically, results obtained from either adult or very young rats were not different. The technique also worked well in the brain tissue of mice, calf and postmortem human brain (data not shown).

To find the optimal conditions, brain slices of different thicknesses were cut, and it was found that a thickness of 300–500 μm was the best for visualization of neural bundles in small animals. 200 μm was a minimal thickness for slices that could be successfully used, as axonal fiber bundles were not viewable in thinner slices. On the other hand, thicker slices were more difficult to view in smaller animals because of the
accumulating volume of white tissue. Considering that in large animal and human brains, large fiber bundles may extend beyond the slice thickness of 400–600 μm, it should be noted that there was no immediate limitation on maximal slice thickness in this technique per se. Depending on the microscope specifications and anatomical organization of the observed brain region, thicker slices (1 mm and more) sometimes served better. Another peculiarity of this method is that fiber architecture can be effectively observed only under low magnification, such as with 2 ×, 5 × or 10 × objectives. Choosing the proper distance between condenser and specimen to provide appropriate illumination was very important. While setting up good dark-field illumination required some practice, obtaining results was not that difficult. Initial successful experiments were carried out in home laboratory conditions. A potential problem might be the solidification of curved sections after methyl salicylate exposure, which would make them difficult to bring into focus, so proper care should be taken to keep slices flattened from the very beginning.

Fig. 1. Fiber tract visualization using dark-field illumination. A, photomontage of the frontal slice of the adult rat brain at the level of the forebrain. B, photo of the horizontal slice of the adult rat brain at the forebrain and thalamus level. C, photo of the spinal cord slice (thoracal segment) of a 16-day-old rat. Abbreviations, ac, anterior commissure; alv, alveus hippocampus; bic, brachium inferior colliculus; bsc, brachium superior colliculus; cc, corpus callosum; cg, cingulum; cu, cuneate fasciculus; ec, external capsule; f, fornix; fi, fimbria; gr, gracile fasciculus; ic, internal capsule; Ifu, lateral funiculus; opt, optical tract; sm, stria medullaris; str, superior thalamus radiation; vhc, ventral hippocampal commissure; vfu, ventral funiculus.
4. Discussion

This report presents a new, extremely simple morphological technique for visualization of axonal pathways in thick sections of the mammalian CNS. The main principle of the method is to make neural tissue transparent in normal light, and then use the ability of axonal pathways to scatter light for observation using dark-field illumination. To the best of the investigator’s knowledge, the developed method is original.

Transparency of the slices was achieved through delipidation with methyl salicylate, which together with other organic solvents such as xylene, chloroform and dimethyl sulfoxide, is known for dissolving neuronal lipids which causes neural tissue to become more transparent (Becker et al., 1991; Hermes et al., 1996; Grace et al., 1985). Interestingly, for this method, xylene, chloroform and dimethyl sulfoxide did not work. The mechanism of axonal fiber visualization in the technique reported here is currently not clear. Dark-field illumination is known to be an excellent means of increasing the contrast of small light-reflecting particles in tissue. Previous use of dark-field microscopy included viewing single neurons filled with horseradish peroxidase (Price et al., 1977) or measuring immunoreactive fibers (Martin et al., 1994).

One of the most attractive features of this method is that the three-dimensional structure of the whole white matter of the brain slice became clearly viewable. This was best realized in brain regions containing axonal bundles running within a surrounding mass of gray matter such as the forebrain (Fig. 1A). Otherwise, massive concentrations of fibers on the surface of the slice prevented viewing underlying fibers. Thus, capability for 3D-visualization was best utilized only in certain brain regions. This technique was employable only in sections thicker than 200 μm and worked equally well for frontal, sagittal and horizontal sections of the brain, and in the spinal cord of both mature and neonatal mammals. The experience showed that the technique was very useful for visualization of the course of axonal pathways studied in electrophysiological experiments in rat brain. Due to its extreme simplicity, the technique can be used in any lab having basic microscopy equipment and a minimal set of reagents. The only major disadvantage is that the technique does not allow the resolution of individual neuronal fibers.

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References

