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A technique for flat embedding and en face sectioning of the mammalian retina for autoradiography

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A method of tissue preparation is described which allows analysis of large areas of the mammalian retina in relatively few 2–5 μm sections, and which permits [^{3}H]autoradiography with good resolution and low background. In contrast to standard methods of paraffin embedding and radial sectioning, we have embedded flattened retinas in glycol methacrylate resin and cut sections parallel to the plane of the retinal layers, i.e. en face. As previously shown (Sidman, 1970; Carter-Dawson and LaVail, 1979a, b), methacrylate embedding results in excellent cellular preservation and allows relatively thin sectioning appropriate for [^{3}H]autoradiography and demonstration of cytological detail. Sectioning en face reduces the number of tissue sections required to survey large areas of the retina, and allows reconstruction of the topography of each retinal layer from serial sections. Thus, studies of retinal topography using [^{3}H]autoradiography can now be more easily compared to studies of retinal topography using the whole-mount technique (see Stone, 1981).

Introduction

In the last decade the analysis of the retina as a flattened whole-mount has undergone a popular revival (for review, see Stone, 1981). A major reason for this resurgence lies in an ever-growing interest in the topographic organization of retinal elements and of the retinofugal pathways. Certain spatial relationships between cells which are self-evident in a retinal whole-mount can only be extracted from radially sectioned material after tedious reconstructions of large numbers of serial sections.

Whole-mounts, however, are not suitable for autoradiographic studies using tritium (^{3}H), since the beta particle emitted by ^{3}H travels a maximum of 3 μm in

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tissue sections (Rogers, 1979). As part of our ongoing studies of neurogenesis in the cat's retina using [^{3}H]thymidine (Polley et al., 1981, 1982; Walsh et al., 1983; Walsh and Polley, 1984) we have developed a method for flattening and embedding the retina in glycol methacrylate resin (GMA), and then cutting large areas of the retina in the approximate plane of the retinal layers (en face). This method is superior to standard paraffin embedding because of the excellent tissue preservation and demonstration of cytological detail (Sidman, 1970; Carter-Dawson and LaVail, 1979a, b). Since glycol methacrylate is softer than most other embedding media (e.g. Epon, Araldite), large block faces, including almost the entire surface of the adult cat's retina, can be sectioned in one block, so that each en face section samples extensive areas of single retinal layers. This technique serves as a bridge between studies of retinal topography using the whole-mount, and studies which require sectioning.

Materials and Methods

Preparing the retina

After the in utero injection of [^{3}H]thymidine into fetal cats of known age (Hickey et al., 1983), normally delivered animals (60–250 days postnatal) were deeply anesthetized and perfused transcardially with neutral 10% formol-saline. The corneas were incised for better penetration of the fixative and the entire head was post-fixed and stored in additional fixative for up to 2 years before processing. The eyes were prepared for histologic study by removing them from the head and dividing the globe at the limbus. Tissues anterior to the ora serrata were discarded and the vitreous was cleaned from the retinal surface. The sclera and optic nerve were dissected and discarded, leaving a cup-shaped structure consisting of the choroid and neuroretina. This cup-shaped structure was flattened by making a number of radially directed relaxing cuts (see Figs. 1–3) and trimming to the ora serrata. The preparation was then floated onto a clean glass slide in saline with the vitreal surface uppermost, and was carefully cleaned of any remaining vitreous or debris (see Stone, 1981).

Embedding the retina

The tissue was then floated onto the shiny side of a circular polycarbonate filter membrane (47 mm diameter, Nucleopore Corp., Pleasanton, CA) and covered with a second filter membrane. The smooth surface of these membranes does not become adherent in subsequent processing, is freely permeable, and is not affected by subsequent dehydration and infiltration. The filter membranes and the enclosed retinal tissues were placed between two flat pieces of polypropylene screen (Small Parts, Inc., 6901 N.E. 3rd Ave., Miami, FL 33138) which were sewn together with cotton thread in order to keep the tissue flat through dehydration and infiltration. The entire 'sandwich' was dehydrated (1 h each in 50%, 70%, 95% and 100% ethyl alcohol) and left overnight in an *infiltrating solution* of GMA (Sorvall, E.I. du Pont de Nemours and Co., Inc., Wilmington, DE).

A 1–3 ml volume of an *embedding solution* was prepared at this time and the mixture was poured into the inverted top of a 60 × 15 mm plastic Petri dish (no. 1007; Falcon, 1950 Williams Drive, Oxnard, CA). Fig. 1 shows the method of preparing a polymerized membrane of methacrylate for the flat embedding of the choroid-retinal tissue. After pouring the unpolymerized embedding mixture into the inverted Petri dish top, the bottom piece was placed in the inverted top and lightly weighted (about 10 g). The fluid GMA is displaced except for that volume contained in the space formed by 3 small plastic protrusions in the inverted Petri dish top (Fig. 1a). The Petri dishes and the enclosed methacrylate were placed in a dessicator jar and allowed to polymerize and harden overnight. The following day, the lower or outer plastic dish was broken away leaving a thin (0.3–0.5 mm thick) polymerized methacrylate membrane adherent to the bottom of the inner dish (Fig. 1a). Excess GMA was trimmed from the sides of the dish.

At this stage, the tissue 'sandwich' was cut open and the flat infiltrated tissue wafer of choroid and neuroretina was carefully removed from the polycarbonate filter membranes. An additional 3 ml of an embedding mixture was prepared and

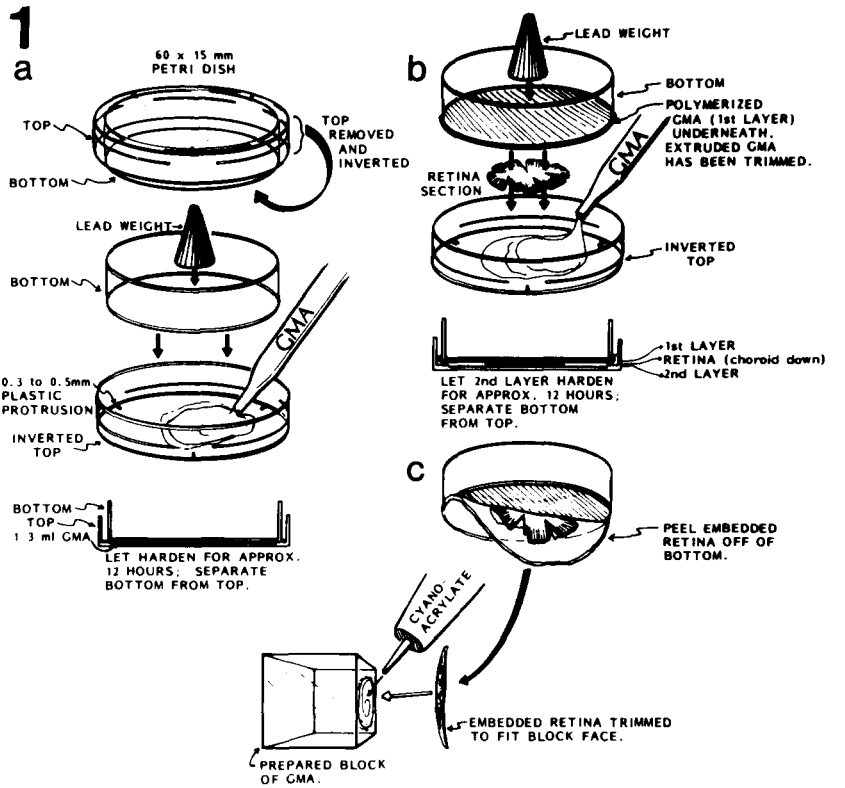

Fig. 1. This series of drawings summarizes the method described for flattening and embedding the retina. a: a plain flat sheet of methacrylate is prepared. b: the retina is embedded against this prepared sheet. c: removal of the retina and preparation of the retina for en face sectioning.

poured into a clean inverted petri dish top (see Fig. 1b). The retinal wafer was placed in this solution with the vitreal surface up, taking care to remove air bubbles. At this point the vitreal surface is easily distinguishable by the presence of the reflective tapetum, which is seen through the transparent neuroretina (see Fig. 2). The previously prepared Petri dish top, with its adherent layer of polymerized GMA, was then carefully lowered onto the wafer in the, as yet, unpolymerized embedding mixture, taking care again to avoid trapping air bubbles. The entire assembly was stored overnight in a dessicator to polymerize. Although a vacuum has been suggested as a means of accelerating the polymerization, we have found that it may cause bubble formation and consequently we avoided the use of a vacuum for the initial stages of embedding. Once the GMA has set, a vacuum is useful to help harden the GMA before sectioning.

Sectioning the retina

On the following day, the outer plastic dish was broken away and the flattened tissue was now enveloped in a sheet of polymerized GMA, approximately 0.6–1.0 mm thick, which could be peeled away from the inner dish (see Fig. 1c). In order to cut en face sections, a block of polymerized GMA was glued to an object holder and was 'faced-off' by cutting a smooth flat surface on a microtome. The resin sheet containing the choroid-retina was photographed or sketched (see Fig. 3), and then was trimmed to an appropriate size so as to match the shape of the prepared block. This sheet was then glued to the prepared block (vitreal surface to block face) with cyanoacrylate adhesive, as shown in Fig. 1. The object holder was then replaced in the microtome and clamped in exactly the same position which was used for preparing the 'faced off' surface.

We have cut serial sections of retinal tissues at 2–5 μm with little difficulty, using either a JB-4A or MT-1 Porter-Blum microtome. Glass Ralph knives, either 25 or 38 mm wide, were prepared on an LKB or Pelco knife-breaker and were mounted with dental wax on an LKB adaptor. Sections were picked up with fine forceps and floated on a 10% solution of ethyl alcohol containing 1–2 drops of ammonium hydroxide per 100 ml of solution. The sections were mounted on gel-coated glass slides and dried on a warming table. We typically cut 100–150 serial sections, 4–5 μm thick, from a block size as large as 25 × 15 mm (Fig. 4), but each cellular layer was contained in less than one-half to one-fourth of these sections. Air-dried slides were processed for autoradiography using standard methods (Rogers, 1979) and stained with 0.1% cresyl violet for 2–4 min. The slides were briefly rinsed in distilled water, air-dried, and coverslipped. Sections have also been stained successfully with 1% methylene blue/1% Azure II in 1% borax.

Results

In en face sections, as in a whole-mount, the retinal landmarks can be located, the retinal quadrants can be accurately defined, and the pattern of autoradiographic label can then be related to these (see Fig. 4). In our own work on ganglion cell

neurogenesis in the cat, we have reconstructed en face plots of large regions of the ganglion cell layer following [^{3}H]thymidine administration to the fetus. These reconstructions are prepared by plotting heavily labeled cells in serial (or alternate) sections and then superimposing these plots. Illustrations of en face plots and further information on how they are constructed can be found elsewhere (Walsh, 1983; Walsh and Polley, 1984).

The combination of the en face plane of section and the excellent cytological preservation reveal the histology of the inner nuclear layer in remarkable detail. An

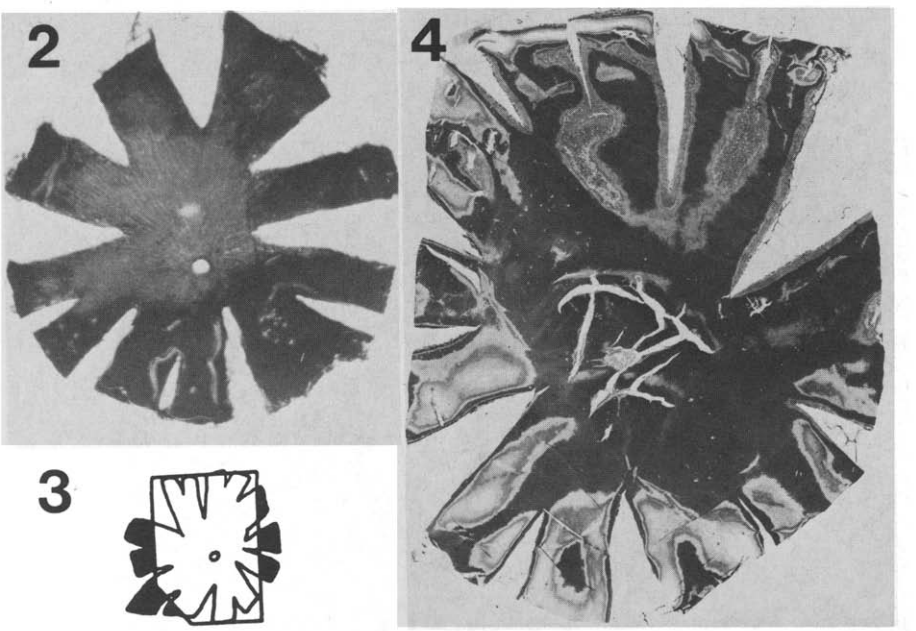

Fig. 2. This photograph shows a cat retina embedded in glycol methacrylate resin. The lighter area in the upper left is the tapetal region, which allows orientation of the retina: superior retina is up, temporal retina is to the left. The round spot represents the optic disc, and a second, upper, light spot in the tapetal region was produced by a tear in the choroid. In order to flatten the retina, several relaxing cuts have been made around the periphery of the retina. ×2.7.

Fig. 3. This drawing shows the retina of a 61-day-old cat. After embedding, the retina was traced, and the region enclosed by the outline was cut out, glued to a block face, and sectioned en face. The circle represents the optic disc. Note that virtually the entire retina could be sectioned as one block. ×1.2.

Fig. 4. This photomicrograph shows a 5 μm section taken in a plane roughly parallel to the retinal layers. This section comes from the block drawn in Fig. 3, and includes almost the entire surface of the retina. The ora serrata can be seen at the top and bottom of the section, and the optic disc is the light-staining spot in the center of the section (cf. Fig. 3). Several tears in the retina near the optic disc occurred during processing, and relaxing cuts were made around the periphery of the retina to allow flattening. This section shows mainly receptor cell nuclei, which stain very darkly, although in a few places the more lightly staining cells of the inner nuclear layer are present. This section is one of 54 serial, 5 μm sections which included the entire neural retina, pigment epithelium, and choroid. Methylene blue/Azure II stain. ×4.

intricate vascular plexus can be seen at the interface of the inner nuclear and outer plexiform layers (Fig. 5). The horizontal cells are embedded within this vascular plexus. In the cat, horizontal cells have been divided into A-type and B-type on the basis of their content of neurofilaments (Wässle et al., 1978). The dendrites of the A-type horizontal cells are shown well because of the low affinity of the neurofilaments for various histologic stains (e.g. cresyl violet). The A-type horizontal cells are especially well shown using a methylene blue/Azure II stain.

In the outer nuclear layer, rod and cone nuclei can be differentiated in terms of their nuclear chromatin patterns (Fig. 6). This has previously been described in the mouse by Carter-Dawson and LaVail (1979a) using radially sectioned, methacrylate-embedded tissue. The cones are found exclusively in the outermost (sclerad)

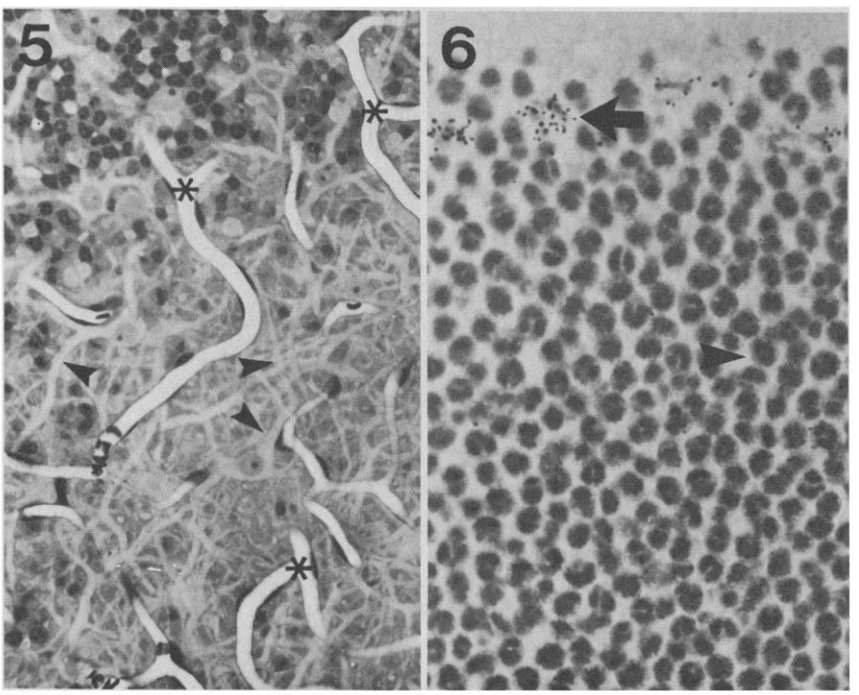

Fig. 5. This photomicrograph shows the outer part of the inner nuclear layer of a cat retina, in a 2 μm en face section, stained with methylene blue/Azure II. Note the larger, pale-staining dendrites of the A-type horizontal cells (arrowheads) which produce a dense overlapping dendritic network (Wassle et al., 1978). Note also the rich capillary network (asterisks) at the interface of the inner nuclear and outer plexiform layers. $\times 700$.

Fig. 6. This photomicrograph shows the outermost portion of the outer nuclear layer (ONL) of the retina of a cat given [^{3}H]thymidine on the thirty-first day of gestation and killed 62 days after birth. Cone nuclei are differentiable from rod nuclei because: (1) they are limited to the outermost part of the ONL (uppermost in this figure); and (2) their nuclei stain more lightly (Carter-Dawson and LaVail, 1979a, b). Note that in this autoradiograph, radioactivity is limited to the cone nuclei (arrow), and is not found over any rod nuclei (arrowhead). Note also the virtual absence of autoradiographic background. $\times 900$.

portion of the outer nuclear layer, and this layering is accentuated by the plane of section, which is oblique with respect to the retinal layers, and which thus increases the apparent vertical dimension of each layer (Fig. 6). Figs. 6 and 7 represent sample autoradiographs (using [^{3}H]thymidine), and show that the embedding method we describe produces autoradiographs with high resolution and very low background.

Conclusions

The method of en face thin sectioning of the glycol methacrylate embedded retina has a number of advantages over conventional methods of radial sectioning of paraffin-embedded tissue. These advantages are: (1) improvement in the resolution of cytologic detail of the cells in the retina; (2) good quality autoradiographs with low background in 2–5 μm sections; and (3) availability of serial sections of extensive areas of individual retinal layers which permit comparison with whole-mount preparations, and which accentuate patterns of inter- and intralaminar details in the cellular layers of the retina.

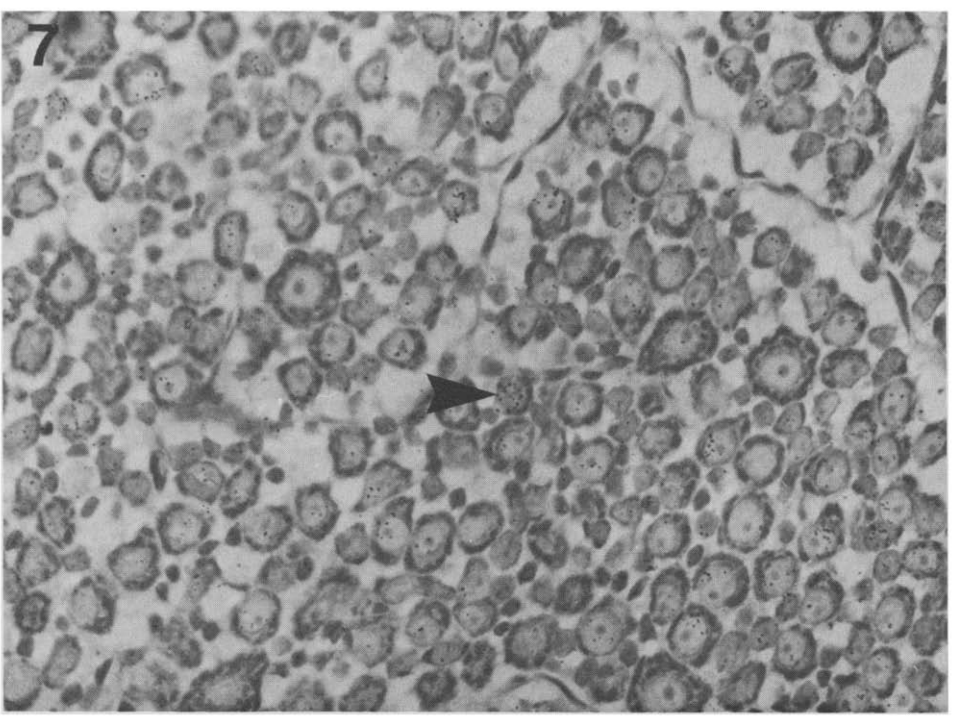

Fig. 7. This photomicrograph shows an autoradiograph of the ganglion cell layer of an adult cat retina which was exposed to [^{3}H]thymidine on the twenty-first day of gestation. This section shows a few heavily labeled ganglion cells (arrowhead) as well as numerous cells which are more lightly labeled. Cresyl violet stain. $\times 700$.

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