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Non-Fluorescent Dye Staining of Primate Blue Cones

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The intravitreal injection in macaque retina of the fluorescent dye Procion yellow can selectively label a specific cone population whose eccentricity distribution and angular separation are consistent with those of the blue-sensitive cones of human and non-human primate retinas. Because at the concentrations used the dye is poorly visible in conventional light microscopy, fluorescence microscopy is required for the observation of the stained cones. In this paper we describe several alternative methods for the staining of blue cones in primate retina, staining that can be visualized in conventional light microscopy and, with some methods, electron microscopy. Invest Ophthalmol Vis Sci 24:1449–1455, 1983

Intravitreal injections of the tissue-reactive, fluorescent dyes Procion yellow M4RS and M4RAN have been used for the selective staining of different retinal structures. At low concentration, Procion yellow stains the extracellular compartment of the outer segment of cones, but it does not significantly stain rod outer segments.[1–4] At higher concentrations, however, Procion yellow also produces the complete intracellular staining of a specific cone population of the retinas of macaques[4] and of other primates (see below). As a result of their characteristic retinal distribution and angular spacing, these selectively stained cones have been identified as the blue-sensitive cones.[4,5]

Because at the concentrations used the dye is poorly visible in conventional light microscopy, fluorescence microscopy is required for the observation of the stained cones. In addition to this requirement, one disadvantage of this method is the fading (or bleaching) of the fluorescence of the stained structures, fading that is produced by the short-wavelength illumination necessary for the fluorescence excitation of the dye. We have developed several alternative methods for the visualization of the blue cones in conventional light microscopy. First, we describe methods for the direct visualization of cells stained by Procion yellow. Second, we describe methods that allow for the substitution of this dye by non-fluorescent, light-opaque dyes that also selectively stain the blue cones.

Visualization of selectively stained cells can be obtained by the differential suprastaining of the tissue with thionin. Although it destroys the fluorescence of Procion yellow, thionin yields a complete and darker staining of Procion-stained structures with a color different from that of other retinal cells that are normally stained by thionin. Visualization of blue cones in conventional light microscopy also can be obtained by injecting Procion yellow with a light-opaque dye such as Procion brown, instead of Procion yellow alone. Since, unlike thionin suprastaining, these mixtures do not destroy fluorescence, the blue cones then can be seen in both fluorescence and conventional light microscopy. Finally, instead of using Procion yellow, selective staining of primate blue cones can be obtained simply by the intravitreal injection of non-fluorescent, light-opaque dyes such as Procion black.[6] Because some of the non-fluorescent dyes contain heavy metals,[7] it is also possible to study blue cones with the electron microscope.

Materials and Methods

The methods for the staining of blue cones by Procion yellow have been described elsewhere.[4] Briefly, a

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solution of Procion yellow M4RAN (Inolex Corp., Glenwood, IL, or Polysciences Inc., Warrington, PA) in distilled water was injected slowly into the vitreous body with a 27-gauge needle inserted behind the temporal limbus; dye concentration and the small injection volume were adjusted to achieve an effective vitreal concentration of 0.25% to 0.30%. Similar results were obtained whether or not the dye solution was passed through a 0.22-μm filter. We used adult baboons (*Chaeropithecus papio*) and macaque monkeys (*Macaca mulatta* and *Macaca irus*) of either sex. The animals were anesthetized lightly with ketamine or with a ketamine–xylazine combination,[8] the cornea and conjunctiva being anesthetized additionally with topical proparacaine; often, pilocarpine was applied topically before the injection of the dye. About 24 hours after the injection, the eyes were fixed for histologic examination. Selective staining of blue cones was unaffected by the method of fixation which could be: (1) immersion of the unopened globe into unbuffered 10% formol-saline alone; (2) with prior transcardial perfusion with the same fixative; or (3) transcardial perfusion and further fixation of the unopened globe by immersion with phosphate-buffered (*p*H 7.4) 3% paraformaldehyde. After 1 hour of immersion, the globe was hemisected and the vitreous body was drained from the posterior half of the eye which was blocked and mounted (vitreal side down) onto glass-fiber filters (type A/E, Gelman Sciences Inc., Ann Arbor, MI). For retinal whole-mounts, the sclera, choroid, and pigment epithelium were removed gently from the neural retina, which then was dehydrated, cleared, removed from the glass-fiber filter, mounted on a glass slide with Diatex (Scientific Products, McGaw Park, IL) and coverslipped. For retinal sectioning, smaller retinal pieces were embedded in Epon 812 resin, JB-4 medium (Polysciences Inc.) or, more rarely, celloidin using conventional embedding procedures.

In the double procion staining method, a solution of Procion brown MX5BR or H3RS (Polysciences Inc.) and Procion yellow M4RAN in distilled water was injected so that each dye had an effective vitreal concentration of 0.175%. In the substitution method, a very fine suspension (15% in distilled water) of Procion black SP-L (ICI of Americas, Wilmington, DE) was injected intravitreally without any filtering to achieve an effective vitreal concentration of 0.75% to 1%. In the latter method, the vitreous humor was stained uniformly after the injection and showed no evidence of fine particulates, indicating that all of the dye entered into solution in the larger volume of the vitreous body (ie, 3 ml for the adult macaque eye). For light microscopy, the retina stained by either method was processed as described above. For electron microscopy of retinas stained with Procion black, the animal was perfused transcardially with phosphate-buffered (*p*H 7.4) 2% paraformaldehyde, 2.5% glutaraldehyde, and 5×10^{-4} M calcium chloride. Retinal pieces were dehydrated in ethanol, embedded in Epon 812, and cut into ultrathin sections.

Thionin suprastaining was used on 5- to 15-μm sections of retina previously stained with Procion yellow M4RAN and embedded either in celloidin or in JB-4 medium. The sections were immersed for 20 minutes in a 0.35% solution of thionin in 0.1 M acetic acid and 0.1 M sodium acetate, at *p*H 3.7; the staining was differentiated in 70% ethanol. Celloidin sections, handled loose throughout the procedure, then were dehydrated, cleared, and coverslipped; JB-4 sections, mounted on glass slides throughout the procedure, were rehydrated, dried on a hot plate at 60°C, and coverslipped. Episcopic fluorescence and conventional light micrographs (Kodak Ektachrome and Kodachrome film) were obtained with Nikon Fluophot® and Zeiss Universal® microscopes; color prints were obtained directly from these micrographs with a Polaprinter copier using Polaroid film type 669.

Results and Discussion

Visualization of Procion-yellow Staining

Figure 1A shows the selective staining of blue cones in the parafoveal retina of the baboon. The cones are stained in their entirety and appear as Golgi-like silhouettes[4] (see also page 259 of reference 2). In the baboon, the stained cones appear to be longer and more slender than those at comparable eccentricity of macaque retina stained by the same method,[4] being generally similar to foveal blue cones of the macaque. The thionin suprastaining of the same radial section of baboon retina is shown in Figure 1B. Here, the completely stained blue cones appear of a reddish-brown color in conventional light microscopy (bright field), and a similar result was obtained for other structures that also were stained by Procion yellow, such as the outer segments of the other cone types (arrows in Figs. 1A–C).[1–4] The inner segments of these other cone types, however, remain unstained by thionin, as is the case when this dye is used alone. It is worth noting that those retinal cell components that normally are stained by thionin, such as cell nuclei, have a color that is different from that of the suprastained blue cones, this difference allowing for an easy distinction between Procion-yellow-stained and non-stained structures.

* Procion yellow MX4R (Polysciences Inc. or ICI of Americas) is supposed to contain the identical dye as the M4RAN variety, but it also contains additional filler substances (ie, urea, salts, paraffin, etc). At the same Procion dye concentrations, MX4R does not yield results with thionin suprastaining as good as those obtained with M4RAN. We have also found poor suprastaining in tissue stained with old batches of M4RAN which, presumably, were stored improperly.

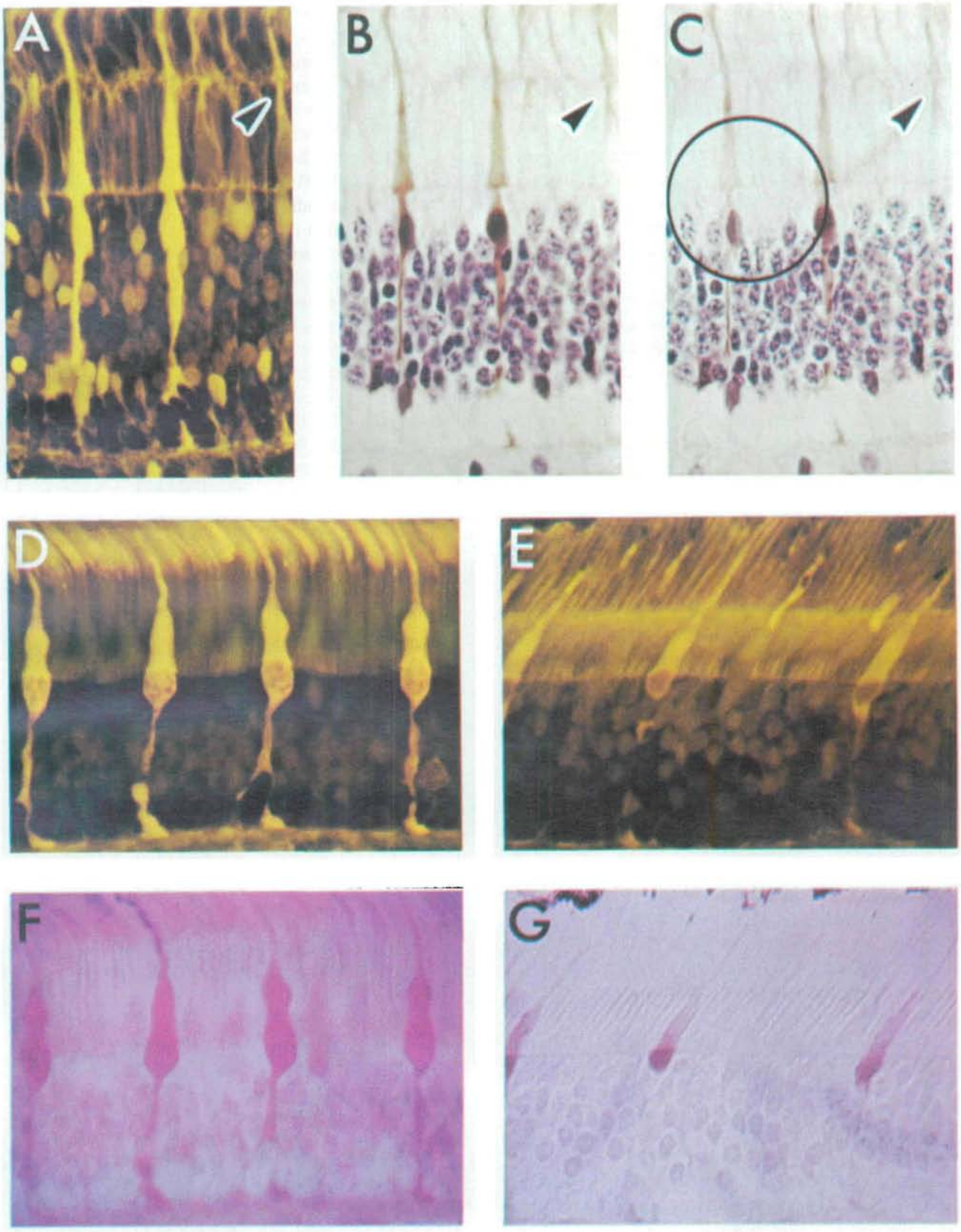

Fig. 1. Radial sections of primate retina stained by the various methods described in the text. **A–C:**Baboon retina stained with Procion yellow (**A**) and suprastained with thionin (**B, C**); encircled region in **C** indicates area exposed to intense UV light for 5 hours; arrowheads point to the stained outer segments of cones. **D–G:** Macaque retina stained with a mixture of Procion yellow and Procion brown H3RS (**D, F**) or MX5BR (**E, G**), seen in fluorescence microscopy (**D, E**) and conventional light microscopy (**F, G**).

It is likely that thionin chemically reacts with Procion yellow. Many Procion dyes contain an anionic sulfate group,[9,10] so it should not be surprising to find binding of a basic dye like thionin. No loss of the suprastaining was observed after many hours of exposure to an intense white light from a tungsten source. While very prolonged exposure to UV light faded the suprastaining reaction product, only a partial fading was observed after a 5-hour exposure to intense UV-blue light from a mercury source, as shown in Figure 1C (encircled region), demonstrating the resilience of the suprastaining. Retinal sections suprastained with thionin revealed no fluorescence due to staining by Procion yellow, Figure 1A having been photographed before the suprastaining of Figure 1B. The absence of fluorescence might be a result of the conversion of the M4RAN dye to a non-fluorescent form by reaction with thionin; alternatively, the (yellow) fluorescence of M4RAN might still remain after suprastaining, but it is quenched by the additional presence of the (blue) thionin dye.

Despite some loss of resolution, the suprastaining has the advantage of providing a resilient, light-opaque labeling that can be used for the study of the distribution pattern and retinal density of blue cones in radial and tangential sections. This is shown in the tangential sections of Figures 2A and 2B, illustrating different retinas stained with Procion yellow, respectively, without and with thionin suprastaining for the extrafoveal retina of the Rhesus monkey.

We found that blue cones also can be visualized in conventional light microscopy by mixing Procion yellow with a light-opaque dye of the Procion family. We have used two Procion brown dyes for this purpose: MX5BR, which is also electron-dense,[7] and H3RS, which is electron-lucent (R. C. Caruso, unpublished observations). We achieved the double Procion staining using similar concentrations of the mixed dyes (see Methods). Figures 1D and 1F show results obtained from the extrafoveal retina of the Philippine (cynomolgus) macaque using a mixture containing the H3RS variety of Procion brown. The double staining of blue cones can be seen in both fluorescence (Fig. 1D) due to Procion yellow and in conventional light microscopy (Fig. 1F) due to Procion brown. As shown in Figures 1E and 1G, similar results also were obtained with the MX5BR variety, although there was more attenuation of the fluorescence of Procion yellow (Fig. 1E) and the Procion brown staining, less uniformly distributed within the blue cones, showed a higher concentration in the nucleus than in other parts of the cell body (Fig. 1G). Because MX5BR is electron-dense, as it contains chromium,[7] the latter mixture presumably could be used for electron-microscopic studies. The double Procion staining also can be used for visualizing blue cones in retinal whole-mounts. This is shown in Figure 2C for a mixture containing the H3RS variety. A disadvantage of this method, at least at the concentrations we have used, is the low contrast of the light-opaque staining produced by either Procion brown variety in mixture, especially MX5BR. To increase the contrast of the color micrographs, Figures 1F, 1G, and 2C were photographed through a custom-made magenta filter (Wratten Y-9495, Eastman Kodak Co., Rochester, NY).

It is worth noting that whereas the type of fixation used did not affect the selectivity of blue cone staining (as all of the methods reported here yielded stained cones having essentially the same retinal distribution and density), the morphology of the stained cones may have been affected by the quality of fixation. For macaque blue cones, this can be observed by comparing Figures 1E and 1D. The former, showing all ellipsoids of normal shape, was obtained from an eye fixed by transcardial perfusion and immersion in buffered paraformaldehyde; the latter, showing swollen ellipsoids, was obtained from an eye fixed only by immersion in unbuffered formol-saline. In addition to fixation artifacts, we suspect that there might be differences among the various tissue-reactive dyes in their intracellular staining effects on cell morphology.

Substitution of Procion-yellow Staining

Blue cones of primate retina can be stained selectively by intravitreally injecting non-fluorescent, light-opaque dyes that can be used in place of Procion yellow.[6] One such dye is Procion brown H3RS, when used at an effective vitreal concentration of 0.37% to 0.40%. (No selective staining was obtained with the electron-dense MX5BR variety at any dosage we have tried.) For substitution purposes, however, we prefer Procion black SPL that stains blue cones with a contrast much higher than that produced by Procion brown H3RS, as shown in Figure 2D. Unlike the case of fluorescent dye staining, Procion black yields little or no background staining of the outer retinal layers in whole mounts, a characteristic that permits the visualization of blue cones in conventional light microscopy throughout large areas of retina, often including the entire retina.

Figures 2E and 2F compare results obtained, respectively, from a tangential section (inner-segment cutting plane) of a retina stained with Procion yellow and from a whole mount (inner-segment focal plane) of a retina stained with Procion black. Even at the low power used for these micrographs, the latter dye (Fig. 2F) yields a clearer visualization of the stained photoreceptors than the former dye (Fig. 2E) despite the differences in tissue thickness. As shown more clearly in Figure 2D, such retinal whole-mounts permit

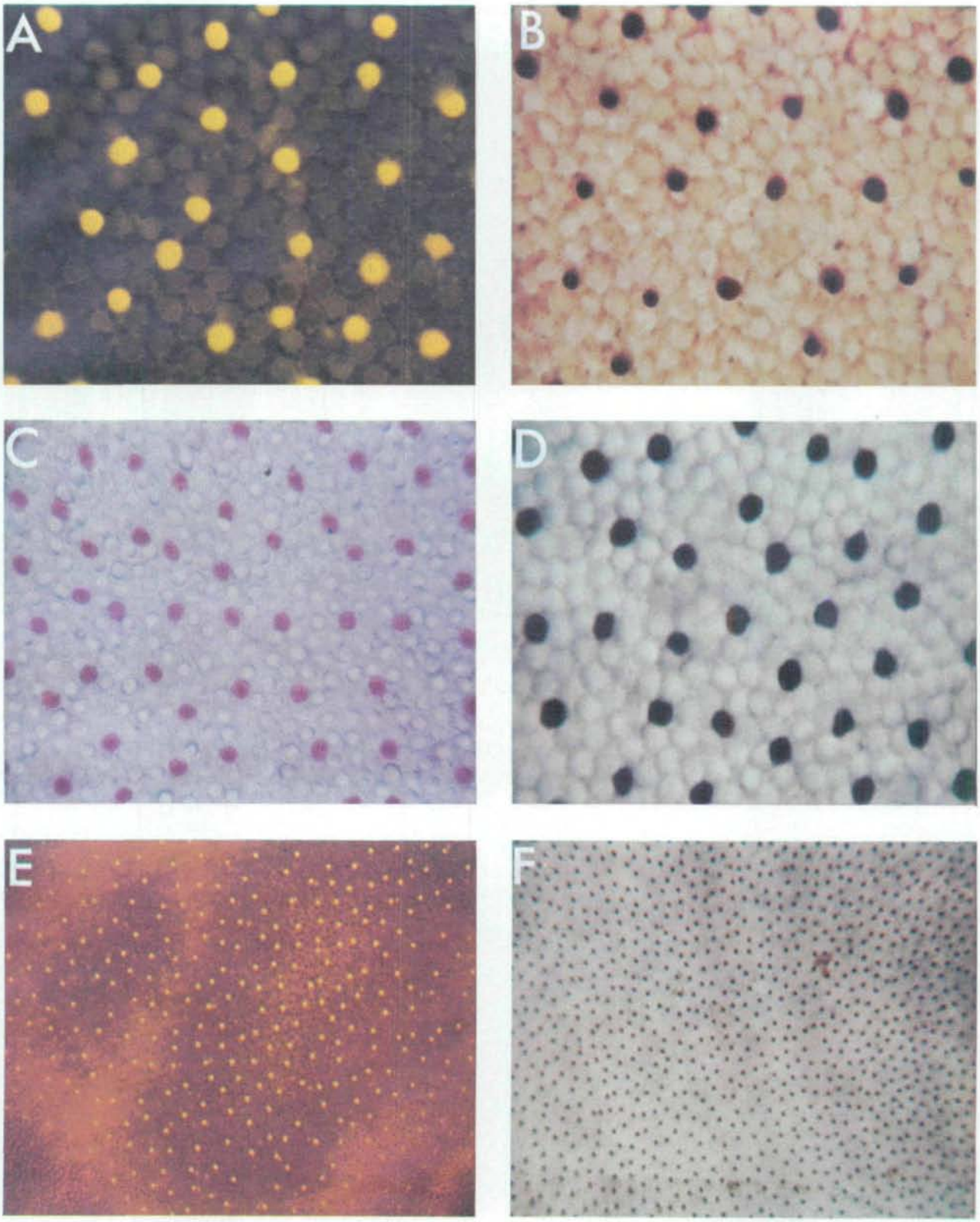

Fig. 2. En face views of macaque retina stained by the various methods described in the text. **A:** Tangential section stained with Procion yellow. **B:** Tangential section stained with Procion yellow and suprastained with thionin. **C:** Whole mount stained with a mixture of Procion yellow and Procion brown H3RS. **D:** Whole mount stained with Procion black. **E–F:** low-power micrographs of extrafoveal retina from tangential section stained with Procion yellow (**E**) and whole mount stained with Procion black; both views at the level of the photoreceptor inner segments (**F**).

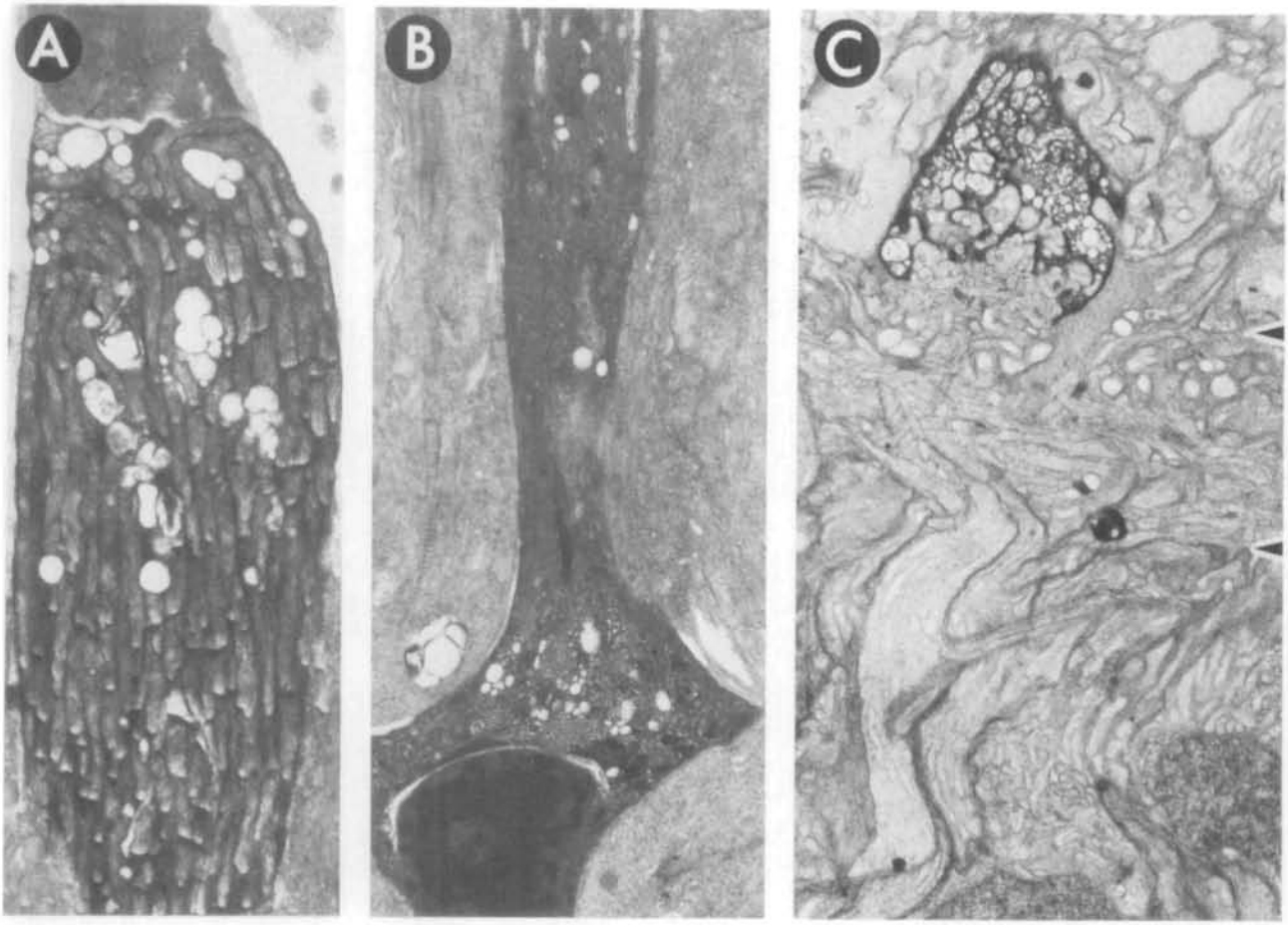

Fig. 3. Electron micrographs of outer layers of macaque retina stained with Procion black. **A:** Blue cone inner segment. **B:** Blue cone nucleus and inner segment. **C:** Blue cone pedicle; arrows delimit outer plexiform layer. Procion black stained structures appear electron-dense, while unstained structures remain electron-lucent.

enough resolution to also distinguish, especially in the regions away from the foveal pit, the unstained inner segments of the other cone types (by slightly closing the iris diaphragm in Köhler illumination).

Data from retinas stained with Procion black have been used for studies of the regularity of the spatial pattern of blue cones[11] (MB Shapiro, SJ Schein, and FM de Monasterio, in preparation), their spatial resolution and aliasing,[12] and of the age and sex dependence of their density counts.[13]

A further advantage of Procion black SPL is the presence of chromium, as indicated by its X-ray energy dispersion spectrum (CE Fiore, SJ Schein, and FM de Monasterio, unpublished observations), so that this dye also should have application for electron microscopy. Indeed, Figure 3 shows electron micrographs of the outer retinal layers of a macaque retina stained with Procion black: the stained blue cones appear electron-dense, while the other photoreceptors (unstained by the dye) remain electron-lucent. Since the pedicles of the stained blue cones can be identified easily (cf. Fig. 3C), this method opens the possibility of studying the ultrastructural connections of blue cones in the outer plexiform layer of primate retina (FM de Monasterio and RC Caruso, in preparation).

Key words: procion dyes, selective staining, blue-sensitive cones, macaque blue cones, baboon blue cones, primate retina

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