

AUTORADIOGRAPHY WITH THE ELECTRON MICROSCOPE

A Procedure for Improving Resolution, Sensitivity, and Contrast

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Autoradiographic techniques for use with the electron microscope have been developed and considerably improved in recent years. A review of this historical development is given by Meek and Moses (14), and a theoretical analysis of the problems involved by Pelc (17) and Caro (4). In this paper, we describe a method for preparing autoradiographic specimens, using a new, fine-grained emulsion and a special, high-sensitivity developing technique. This procedure gives higher resolution, sensitivity, and specimen contrast than previously available.

Fine-grained emulsion: The size of the undeveloped silver halide crystal and the thickness of the emulsion are among the limiting factors in autoradiographic resolution. Ideally, one wants a fine-grained emulsion coated in a closely packed monolayer.

The Ilford L4 nuclear track emulsion with a silver halide grain size of 1200 to 1600 Å is most frequently used for electron microscopic autoradiography. The Gevaert (Scientia Nuc. 307) emulsion has finer grains (700 Å), but is reported to be difficult to handle (7). We obtained an experimental emulsion from Eastman Kodak¹ with an undeveloped grain size of 300 to 500 Å. (Fig. 4 compares the grain size of this emulsion with that

¹We thank Drs. J. Spence and A. G. Millikan of Eastman Kodak, Rochester, New York, for making this emulsion available to us. It is now commercially available as Nuclear Track NTE. (Sensitized Products Division, Eastman Kodak, Rochester, New York)

of the Ilford L4.) The Kodak emulsion has low light-sensitivity (can be handled in yellow-green safe light Wratten filter OA) and has negligible background under the developing procedures described below. As presently supplied (Kodak nuclear track emulsion NTE), the emulsion has too much gelatin for making thin layers of closely packed silver halide crystals. To reduce the gelatin content, 10 cc of water are added to 1 gm of emulsion which is dissolved in a water bath at 60 to 70°C. The emulsion is then centrifuged until the supernatant is clear. To facilitate the separation of the silver halide from the gelatin, the rotor of the centrifuge is first heated with a hot air gun. After centrifugation, the bottom of the centrifuge tube is chilled briefly, and the supernatant discarded. The remaining concentrated emulsion is reheated and diluted with 1 to 4 cc of water per gram of the original emulsion. The exact amount of water added must be determined empirically depending on the emulsion thickness desired. To do this, test slides are coated with a layer of emulsion after successive dilutions until the desired thickness is obtained (Table 1). Very uniform emulsion layers (Fig. 4) are made as follows: a few drops of the rediluted centrifuged emulsion (kept at 60°C) are placed with a medicine dropper onto the slide held horizontally. The emulsion is then drained, and air dried in a vertical position. Tissue sections are first mounted on microscope slides (see specimen preparation), and then coated with emulsion in the same manner (Fig. 1 B).

Emulsion thickness: Emulsion layers on glass slides

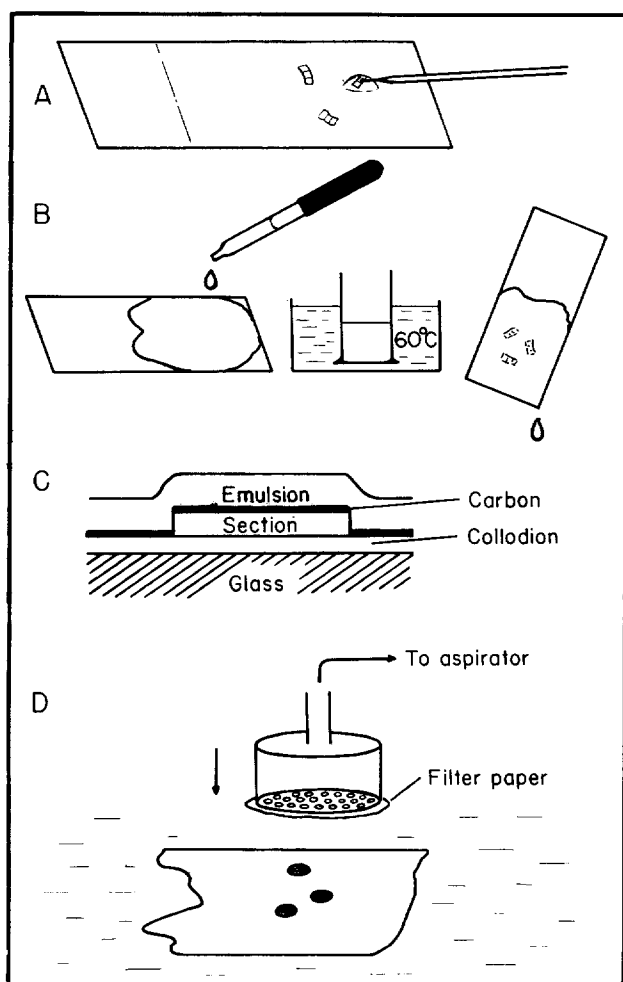


FIGURE 1 Schematic of specimen preparation procedure: *A.* The sections are transferred to a collodion-coated slide, stained, and vacuum coated with carbon. *B.* Emulsion, which is kept at 60°C, is dropped onto the slide held horizontally. Then it is drained and dried in a vertical position. *C.* The different layers which build the specimen "sandwich." *D.* After storage in helium, the slides are developed and the specimen "sandwich" stripped onto water. Grids are placed over the sections and the "sandwich" is sucked onto a filter plate which is covered with wet filter paper.

show, in reflected white light, interference colors which depend on their thickness. Examination with the electron microscope showed that a layer of the centrifuged Kodak NTE emulsion with a silver interference color is a monolayer as seen in Fig. 4. Interferometric measurements showed that this is a uniformly thick layer of 600 Å. In a purple layer the grains are considerably overlapped. That film is roughly equal in thickness to a monolayer of the Ilford L4 emulsion (1500 Å). Table I gives emulsion thickness measurements of Kodak NTE correlated with interference colors. To estimate emulsion thickness over any individual ribbon before developing, one can judge the interference colors in the yellow safe light, where they appear as intensity differences. For higher accuracy, the interference colors of the emulsion over

the section can be observed in white light without introducing background after the specimen has been developed, stopped, washed and dried, but before it is finally fixed. The interference colors remain unchanged after development with developers that do not attack unexposed silver halide—such as Dektol or the Elon-ascorbic acid developer (8) described below.

Development: Sensitivity in autoradiography can be measured by the number of developed grains produced per electron hitting the emulsion. This depends first on the probability that a latent image is formed, and secondly on the developing procedure since the critical size for development of a latent image varies for different developers. With radiation from tritium and silver halide crystals of over 100 Å, the first factor is not yet a limit to

sensitivity (18). We performed experiments with emulsion layers which were irradiated with known doses of 5 and 10 kev electrons² or exposed to tritium, as well as on emulsion layers over hot tissue sections, to evaluate various developing procedures. When the Kodak NTE emulsion is developed with Dektol (diluted 1 to 2) for 1 minute at

TABLE I

Interference Color-Thickness Scale for Sections (16, 3) and Centrifuged Kodak NTE Emulsion

Emulsion thicknesses were measured with a Nomarski interferometer; interference colors (sections on water; emulsion on glass) seen in white light.

Kodak NTE Emulsion centrifuged	Interference colors	Section
500 MONOLAYER or 600A	GRAY	500
	SILVER	
	GOLD	1000
	COPPER	1500
	PURPLE	2000
	BLUE	
Emulsion and section thickness in A		

24°C, filamentous silver grains, approximately 800 to 1400 Å, with negligible background (less than one grain per 50 μ^2) are obtained (Figs. 2 and 5). For comparison, the Ilford L4 emulsion developed with Microdol X (5, 18) for three minutes has developed grains of approximately 2,000 to 4,000 Å (Fig. 3). The sensitivity of these two emulsions when developed this way is comparable. One developed grain is obtained for 12 electrons hitting a monolayer of the emulsion. To get this sensitivity in actual autoradiograms, however, two factors that decrease the developability of the latent image have to be controlled. The first factor is

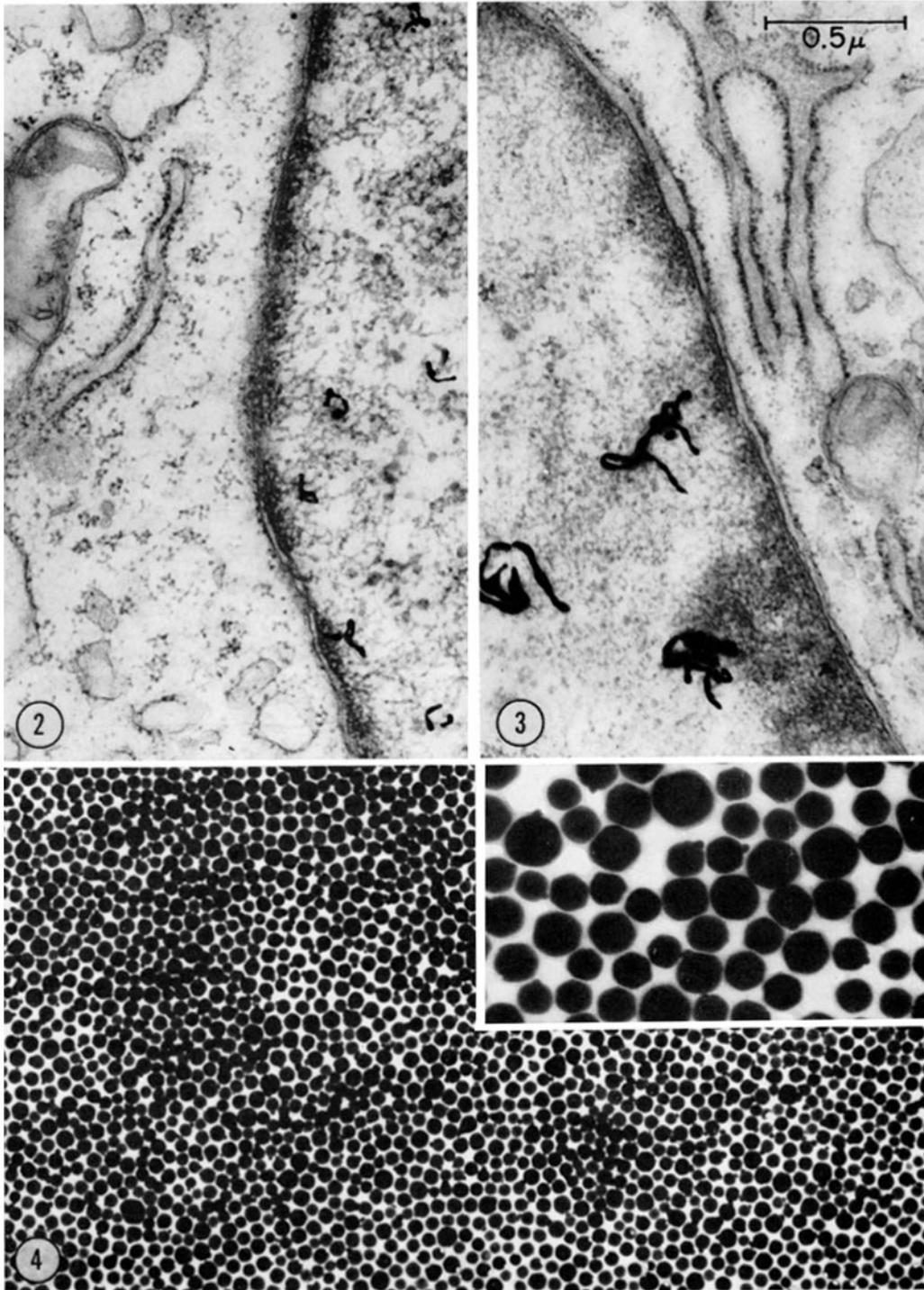
² Slides were irradiated in a modified RCA EMU 3 electron microscope. The intensity of the irradiation was measured with a Faraday cage. Low exposures (3 to 10 electrons per μ^2) were used. Thus the probability that any single silver halide crystal was hit more than once is negligible.

slow oxidation by air of the latent image (19). An exposed layer of Kodak emulsion when stored in air has a 60 per cent decrease in developed grains (Dektol developed) during a 2-month period. This effect is eliminated by storing the emulsion in helium. There is no similar loss in sensitivity in a layer of Ilford L4 emulsion (Microdol X developed). The second factor is a loss of sensitivity when the emulsion layer is in direct contact with a histological section (tissue fixed in OsO₄). In the Kodak emulsion, this loss in sensitivity is very strong even when the relatively coarse-grained Dektol developer is used. In the Ilford emulsion, the effect is more marginal, and a loss in sensitivity is seen mainly when fine-grained development—for instance, with paraphenylenediamine—is used (2). If a 30 to 60 Å carbon layer is evaporated over the biological section before coating with emulsion, it protects the latent image from oxidation by the biological section and raises the sensitivity to that found in isolated emulsion layers.

Fine-grained development of Kodak emulsion: Some standard fine-grained developers such as paraphenylenediamine (15, 5, 2) or Elon-ascorbic acid (8) failed to give adequate sensitivity for the Kodak emulsion. Fine-grained development with high sensitivity is possible, however, after gold "latensification" (11, 12, 9). This is a procedure which deposits metallic gold onto the latent images before the actual development. For gold latensification, 0.5 cc of a 2 per cent stock solution of gold chloride (AuCl₃·HCl·3H₂O) is diluted to 10 cc, and 0.125 gm of potassium thiocyanate is dissolved in it. Then 0.15 gm potassium bromide is added, and the solution diluted to 250 cc. This gold thiocyanate solution is unstable, and we used it only for 1 day. An exposed slide is first dipped in water and then soaked in a 1 to 20 dilution of the gold thiocyanate for 30 seconds, washed, and then developed. The developer consists of 0.045 per cent Elon (Metol), 0.3 per cent ascorbic acid, 0.5 per cent Borax, and 0.1 per cent potassium bromide (8). Development was done at 24°C. (ascorbic acid obtained from Fisher Scientific Co.)

The developed grain size strongly depends upon the developing time and the age of the developer. The developer is unstable and decays very rapidly during the first few hours after it is made, but much more slowly thereafter. When the developer is 5 to 48 hours old, an 8-minute development results in developed grains of roughly 500 Å (Fig. 6).

Sensitivity is increased by gold latensification as



a function of solution strength and of soaking time. The sensitivity with the above procedure is a fourfold increase over that obtained with Dektol alone—one developed grain is obtained for every three electrons hitting the emulsion monolayer. The background in unexposed emulsion layers remains negligible however,—1 grain per $30 \mu^2$. Fine-grained developing produces a certain number (approximately 15 to 20 per cent) of clusters consisting of 2 to 3 small developed grains which were seen even when very low exposures were used. The centers of the grains of these clusters were usually contained within a circle of diameter 600 A (roughly the diameter of one silver halide crystal). Such a cluster must be considered as a single grain.

Gold latensification also increases sensitivity when other developers are used; the strength of the gold solution and the soaking time have to be adjusted for each developer, however. A three- to fourfold increase in sensitivity is produced when gold latensification is combined with Dektol development. The developed grain size remains the same. In this case, the gold thiocyanate solution is diluted 1 to 40 and the emulsion soaked in it for 10 seconds before development.

Specimen preparation: Steps in specimen preparation are depicted schematically in Fig 1. A ribbon of thin sections embedded in either methacrylate or epoxy resin, is placed onto a drop of water on a collodion-coated slide (Fig. 1 A). Care must be taken not to tear the collodion film, which happens if the collodion is touched while wet. The water drop is drained off, and the sections are dried. It is recommended to mark the location of the sections on the back of the slide. More than one ribbon may be placed on the slide. For staining, the slide is put in a petri dish, a few drops of stain placed over the sections, and the dish covered. After the staining period, the drops of stain are

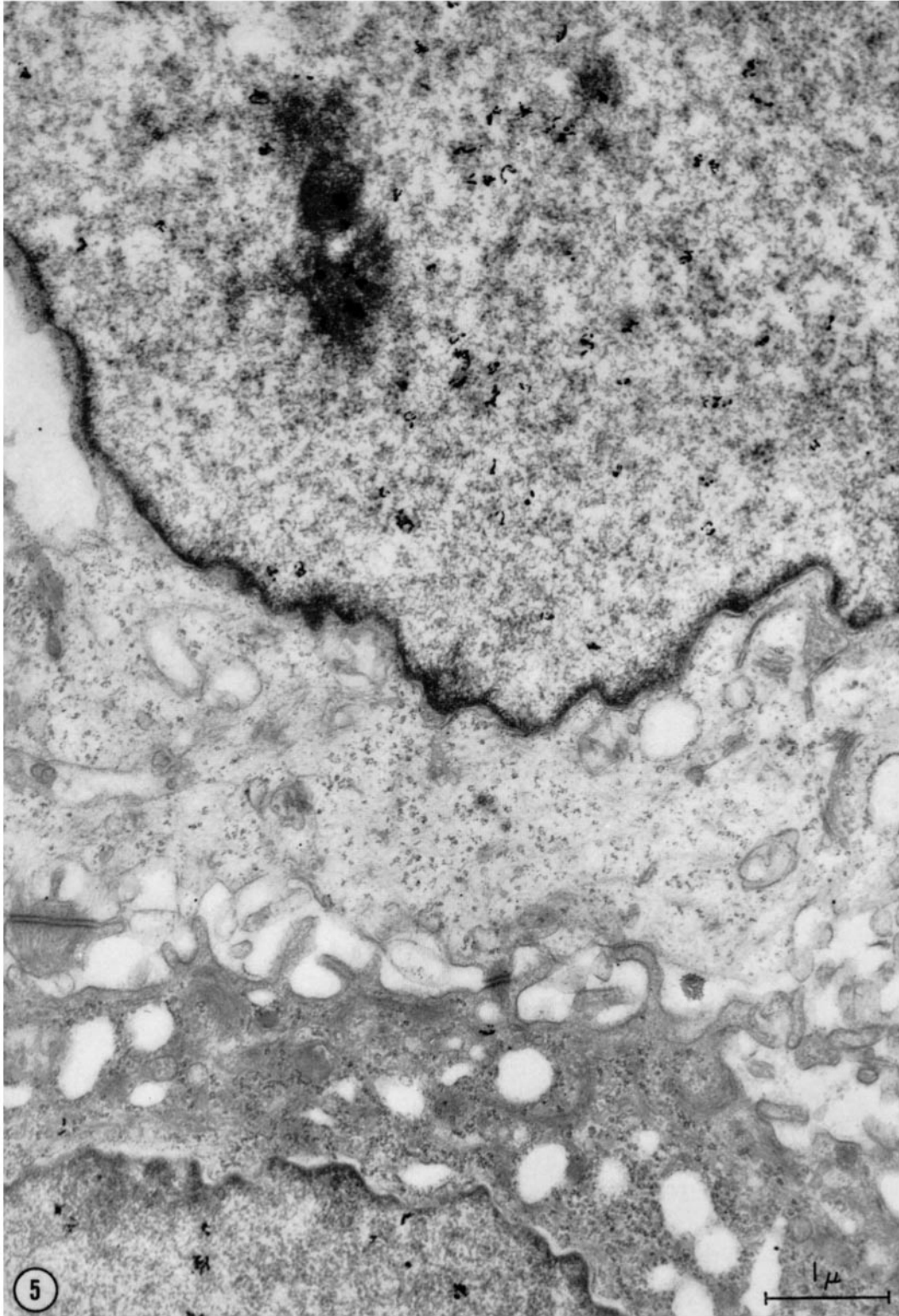
quickly flushed off. We obtained satisfactory results when staining with uranyl acetate for 2 to 5 minutes followed by Reynold's lead citrate (22) for 8 to 10 minutes. Overstaining should be avoided. The sections are then first vacuum coated with a 30 to 60 A carbon layer, and then coated with emulsion as already described. After exposure, developing is performed in a series of beakers—the sequence consisting of: developer; distilled water rinse; 3 per cent acetic acid stop bath for 10 seconds; distilled water rinse; Kodak acid fixer for 1 minute; and several distilled water rinses. The sandwich (consisting of collodion, stained section, carbon layer, and developed emulsion) (Fig. 1 C) is then stripped onto a water surface, grids are placed over the individual ribbons, and the sandwich picked up. A device for doing so is seen in Fig. 1 D. To facilitate stripping the collodion, the slide may be soaked in distilled water for 15 minutes or longer after being fixed, preferably without the slides having first been allowed to dry.

Evaluation of these preparatory procedures: All the steps of this procedure are performed while the tissue sections are on a glass slide. The tissue is not transferred to grids until all the photographic processing is complete. This prevents any interaction between the metal grids and emulsion, and results in cleaner specimens (see also reference 18). Furthermore, since the specimen on glass is completely flat, and the carbon layer provides equal surface properties over the whole specimen, the result is a very uniform emulsion layer. The carbon layer has two additional functions. It protects the latent image from destruction by the biological section as described above, and it protects the stained section from a marked destaining effect of the developing fluid (see also reference 13). Staining tissue sections before coating with emulsion avoids the need to enhance tissue contrast by specimen manipulations (staining or gelatin re-

FIGURES 2 and 3 Mesenchymatous cells from regenerating limb of adult *Triturus* (23). The nuclei of these cells were labeled with H^3 -thymidine (newts injected with $150 \mu\text{c}/\text{gm}$ body wt.). Tissue was fixed in OsO_4 , embedded in methacrylate, stained with lead, and covered with a 50 A carbon layer before coating with emulsion. Fig. 2, Emulsion: Kodak NTE. Development: Dektol for 1 minute at 24°C . Fig. 3, Emulsion: Ilford L4. Development: Microdol X for 3 minutes at 24°C .

Both figures, approx. $\times 40,000$.

FIGURE 4 Closely packed monolayer of centrifuged Kodak emulsion (silver interference color) prepared and coated as described in text. Compare grain size with that of Ilford L4 emulsion (insert), approx. $\times 40,000$.



moval) after photographic processing (10, 20, 21). We found that gelatin removed can introduce dirt and cause removal of developed grains, especially if these grains are small (see also 5, 7, 14). If the stained section is protected from the developing fluids by a carbon layer, the result is a very high contrast specimen, with emulsion gelatin intact (Figs. 2, 3, 5, 6), even when very thin sections (<400 Å—interference color gray) are used (Fig. 6). The high contrast is also obtained when Epon is used as the embedding medium.

The above procedure further provides a closely packed emulsion layer of known thickness, and small developed grains with high sensitivity. A detailed quantitative analysis of resolution and sensitivity will be presented in a second paper, (see also 6).

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FIGURE 5 Epithelial cells from regenerating limb of *Triturus* labeled with H³-thymidine and processed as in Fig. 2 and 3. Emulsion: Kodak NTE. Development: Dektol 1 minute at 24°C.; approximately $\times 18,500$.

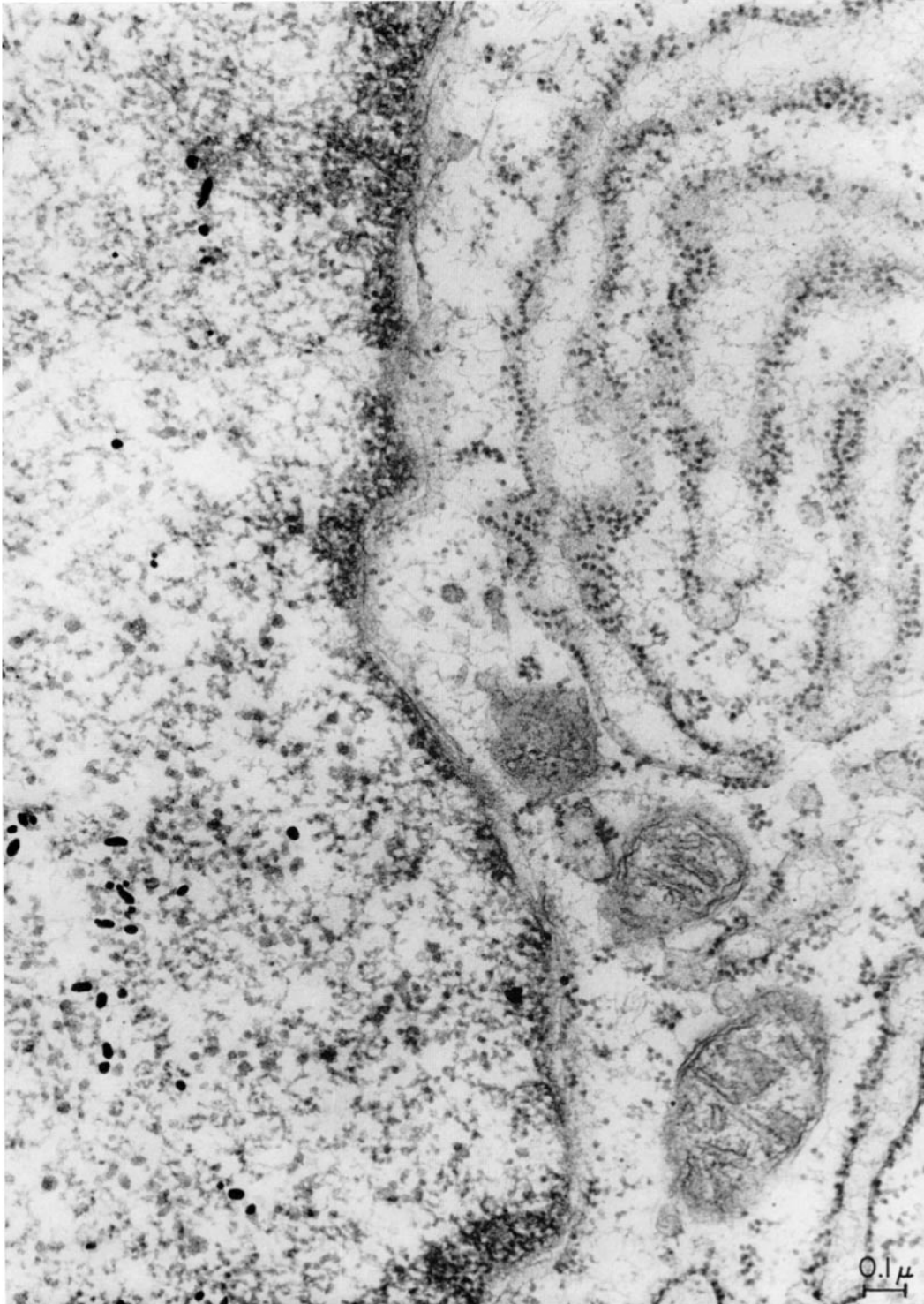


FIGURE 6 Mesenchymatous cells from regenerating limb of *Triturus* labeled with H³-thymidine. Tissue section (approximately 400 Å thick; gray interference color) was double-stained with uranyl acetate followed by lead, and covered with a 50-Å carbon layer before coating with Kodak NTE emulsion. Note small, dense developed grains in nucleus. Development: Auric thiocyanate "gold latensification"—followed by ascorbic acid development. Approximately $\times 60,000$.

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