PROCEDURE FOR EMBEDDING IN SITU SELECTED CELLS CULTURED IN VITRO

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Ultrastructure studies of cells in vitro are greatly facilitated when the procedures used permit combined light and electron microscopy of selected cells or cellular components. The methods currently available for such studies are designed primarily for pre-selection of cells and are not suitable for high resolution light microscope observation. Such techniques are further limited because they require special treatment, such as growing cell monolayers on carbon films (1).

The present report describes a simplified flat-face embedding method which can be used to study individual chromosomes, nucleoli, or other cellular components with both the light and electron microscope. The method is now used routinely in our laboratory and has several advantages over other such techniques: 1, cells may be grown under routine culture conditions; 2, cells or cellular components may be examined and photographed under an oil immersion objective followed by subsequent thin sectioning and examination with the electron microscope; and 3, instead of only a few cells, thousands of cells may be prepared at one time.

METHOD

Mammalian tissue culture cells were grown in Falcon plastic T-30 flasks in McCoy's 5a medium supplemented with 20% fetal calf serum. Fixation was accomplished in situ by the method of decanting the growth medium and adding 3% phosphate-buffered glutaraldehyde (2). After 1 hr, the cells were washed in buffer and postfixed in 1% OsO_4 for 30 min. After fixation, embedding is accomplished by the following schedule; in all steps the original culture flask is used:

1. Wash thoroughly in three changes of distilled water.

2. Pre-stain by covering cell monolayer with filtered 2% aqueous uranyl acetate for 30 min.

3. Wash in two changes of distilled water.

4. Dehydrate either at room temperature or in the cold by the following schedule:

35% ethanol for 10 min.
50% ethanol for 10 min.
75% ethanol for 10 min.
90% ethanol for 10 min.
90% hydroxypropyl methacrylate (HPMA). Three changes over a period of 15 min.
95% HPMA for 15 min.
97% HPMA for 15 min.
2 parts HPMA:1 part Luft's Epon 812 for 15 min.
1 part HPMA:1 part Luft's Epon 812 for 15 min.
1 part HPMA:2 parts Luft's Epon 812 for 30 min.
Pure Luft's Epon, three changes for 10 min. each.

5. Drain off Epon until a thin layer about the thickness of a cover glass is left (just enough Epon left to cover the cell monolayer).

6. Burn holes in top of culture flask with heated glass rod or wire (Fig. 1 *a*). Leave flask overnight in 37°C oven. Transfer to 60°C oven for 24 hr for final polymerization.

7. When polymerization is completed, the top of the flask may be cut away, leaving a thin plastic sheet containing Epon in which the cells are embedded and the bottom of the plastic flask. The total thickness should not be greater than that of a glass slide with cover slip (approximately 1 mm thick).

8. The plastic sheet can then be placed under a phase microscope and the cells examined with an oil immersion objective (Fig. 1 *b*). Cells to be sectioned for electron microscopy are marked by scoring a circle around them with a Leitz diamond slide marker or other sharp object.

9. The scored area is bored out with a cork borer of slightly larger diameter, producing a small disc (Fig. 1 *c*). The disc has Epon on one side and the bottom of the plastic container on the other.

10. The plastic container side of the disc is glued to an old Epon capsule with epoxy cement (Fig. 1 *d*). After the glue hardens, the disc is trimmed and sectioned with either a glass or diamond knife. The Epon's being slightly thicker than the cell layer permits the microtomist to position the knife and start cutting acceptable sections before the cell layer is reached.

11. Sections are picked up on grids and stained with uranyl acetate followed by lead citrate.

An alternative method of examining the embedded cells is to separate the Epon sheet from the plastic container by freezing the flask on dry ice. If the bottom of the container is cut out and placed on a block of dry ice for about 10 min, the Epon plate can be snapped free of the container, due to differences in their expansion coefficients. The cell monolayer

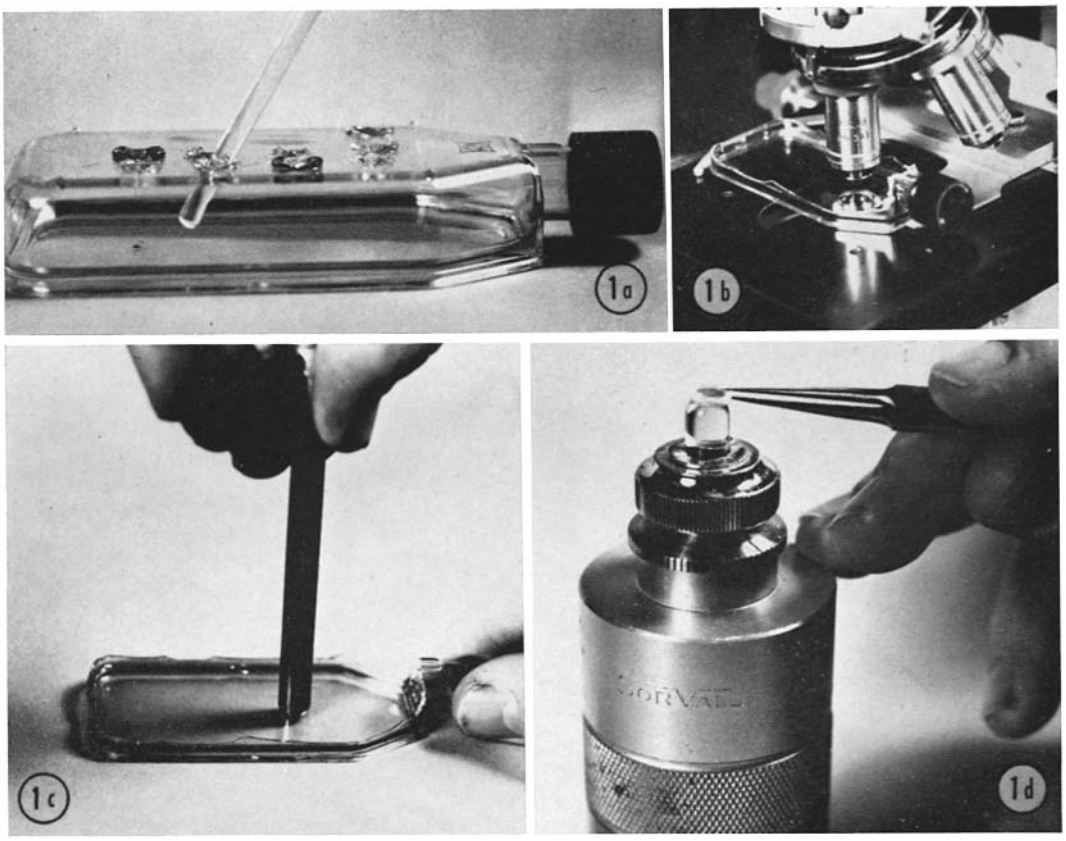

FIGURE 1 Stages in specimen preparation. *a*, A heated glass rod is used to burn holes in the top of the plastic T-30 flask after the final Epon mixture has been added to the cell monolayer. *b*, After the Epon has polymerized, the top of the flask is torn away and the cells examined under phase optics. If the Epon layer is thin enough, the oil immersion objectives may be used. *c*, When a particular cell is located, the area is marked and bored out with a cork borer. *d*, The small disc produced by the cork borer is glued, plastic side down, to an old Epon capsule. After the glue hardens, the block is carefully trimmed around the selected cells and placed in a microtome for sectioning.

will remain in the Epon plate and can be examined, marked, cut out, and mounted on an old Epon capsule for sectioning. By gluing the Epon plate so that the cell monolayer faces the knife edge, one can begin sectioning the cells immediately without cutting through a layer of Epon as previously described.

RESULTS AND DISCUSSION

The primary requirement for using Luft's Epon mixture (3) for embedding cell monolayers grown on a plastic surface is that the propylene oxide step be eliminated since the plastic is soluble in this compound. Initially, this step was avoided by proceeding through a graded series of Epon: ethyl alcohol mixtures until the alcohol was replaced by the pure Epon. Unfortunately, prolonged exposure of the cells to the higher alcohols resulted in excessive leaching of lipid-containing organelles. For this reason, it was necessary to utilize the water-miscible resin hydroxypropyl methacrylate (HPMA) as a dehydrating agent in a manner similar to that developed by Vatter and Zambernard (4). Since HPMA (without catalyst) as a final dehydrating agent results in excellent preservation of cytoplasmic membrane systems, it is now used routinely in all preparations.

Fig. 2 presents a light micrograph and an electron micrograph of the same female rat kangaroo cell in late prophase. The entire chromosome

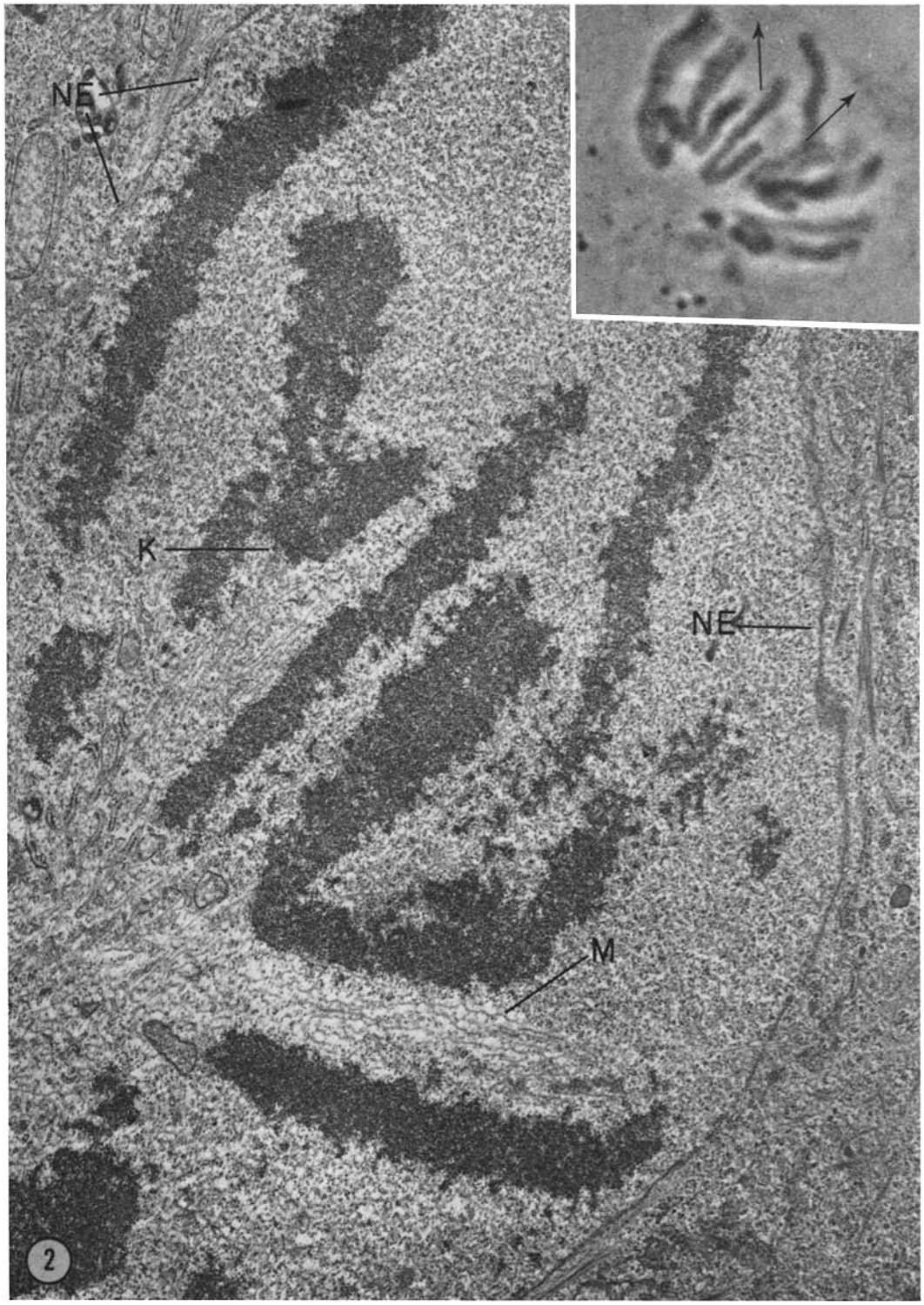

FIGURE 2 Comparative light and electron microscopy of the same prophase nucleus. The inset shows portions of the complete chromosome complement of the female Tasmanian wallaby cell photographed under the oil immersion objectives. The arrows in inset indicate faint outlines of the nuclear envelope. The electron micrograph shows a single section of the same nucleus. Note the excellent preservation of the partially disrupted nuclear envelope (*NE*), microtubules (*M*), and kinetochores (*K*). The chromosomes were undergoing prometaphase movement. × 10,500. Inset, × 1,800.

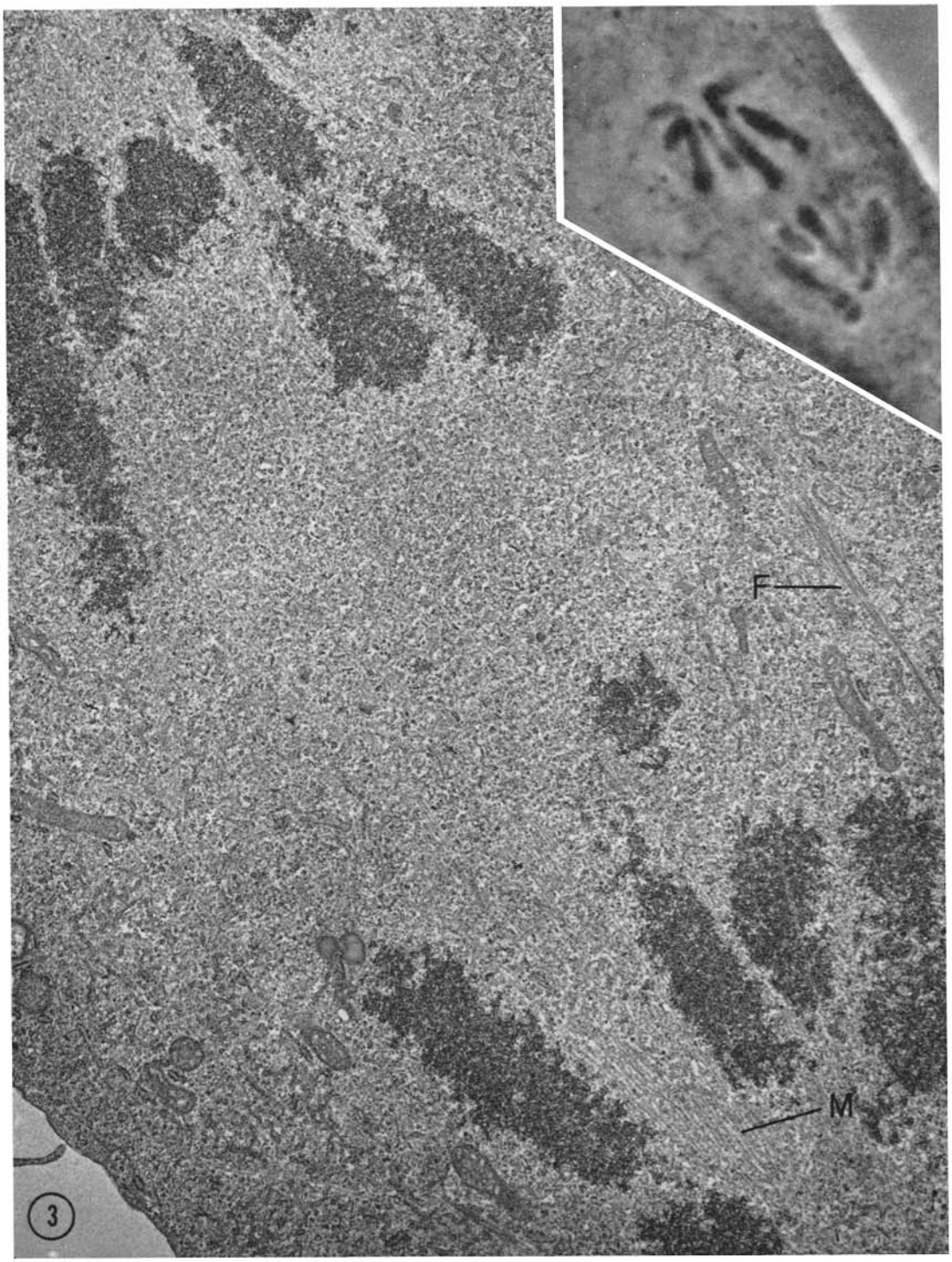

FIGURE 3 Inset shows light micrograph of a cell in anaphase. The corresponding electron micrograph shows portions of the same chromosomes. Note the presence of microfilaments (*F*) and microtubules (*M*). The cytoplasmic organelles and ground substance are well preserved. × 10,000. Inset, × 2,000.

complement (2n = 12) is visible in the light micrograph. Also, remnants of the nuclear envelope are faintly evident. Chromosomes and spindle tubules, as well as portions of the nuclear envelope, are well preserved in the electron micrograph. An anaphase cell is shown in Fig. 3. A group of chromosomes can be seen converging on each pole in the light micrograph inset. Portions of the same chromosomes are seen in the electron micrograph. Note the excellent quality of preservation of both cytoplasmic and chromosomal structures.

The present method provides a simple embedding procedure which is extremely useful for the study of cell structure in vitro. In addition to the excellent quality of preservation at both light and electron microscope levels, the technique assures more reliable interpretations of thin sections in the electron microscope. The ability to preselect cells at particular stages in the mitotic cycle or to study individual cell components of known orientation by serial sectioning should provide much more meaningful information at the ultrastructural level. Application of the method has already made it possible to identify particular chromosomes by their classical morphological features and to study the ultrastructure of specialized regions such as kinetochores, telomeres, and nucleolus organizers (5; T. C. Hsu, B. R. Brinkley, and F. E. Arrighi, in preparation).

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