NUCLEAR-CYTOPLASMIC RELATIONSHIPS IN HUMAN CELLS IN TISSUE CULTURE

V. A Method for Embedding Enucleate Cytoplasm for Electron Microscopy

JULIE MICOU, CARTER C. COLLINS, and T. TIMOTHY CROCKER. From the Cancer Research Institute, the Research and Development Laboratory, and the Department of Medicine, University of California Medical Center, San Francisco

Several methods have been developed for *in situ* embedding of tissue culture cells grown on glass. Borysko and Sapranauskas (1) first reported a technic for embedding whole colonies of cells for sectioning in a plane perpendicular to the glass surface. Howatson and Almeida (2), using a modification of Gay's method for smears (3), embedded cells by inverting gelatin capsules, filled with partially polymerized methacrylate, over selected areas of the slide, producing blocks which could be sectioned parallel to the surface of the glass. The procedures described by Nebel and Minick (4) and by Bloom (5) allow more accurate orientation of capsules over specific cells, but are limited by their equipment to embedding only one field of cells at a time. Nishiura and Rangan (6) recently described a stand constructed for embedding multiple sites with some degree of specificity, and Latta (7) developed a method allowing selection of completely random sites for embedding cultures grown in Petri dishes.

Studies by electron microscopy on the effects of enucleation on the morphologic structure of cytoplasm required modification of these methods to permit more flexible independent positioning of small capsules for simultaneous embedding of a number of randomly distributed single enucleate fragments. As many as 15 to 20 fragments can be produced in one culture vessel (8, 9), but for orientation in sectioning only 1 or 2 should be embedded per capsule.

The technic described in this report utilizes No. 4 gelatin capsules and a magnetic mounting holder. It allows rapid, accurate location and embedding of single cells or fragments in as many as eight capsules per culture.

MATERIALS AND METHODS

Magnetic Mounting Holder

The holder (Fig. 1) consists of a nonmagnetic base plate, 10 by 8 cm, with a circular Alnico V magnet, 2.1 cm in diameter and 1.5 cm high, attached in the center, and four upright posts of angle iron, 4 cm high. The posts, one of which is movable, are placed to accommodate the corners of the slide bearing cultured cells or enucleate fragments. The movable post, attached by a spring to the adjacent permanently mounted post, is mounted in a slot with a guide pin. By means of the guide pin, the post can be adjusted to compensate for irregularities between glass slides, or locked in place to hold the slide at a fixed distance above the gelatin capsules used for embedding. A steel plate slightly smaller than the glass slide is

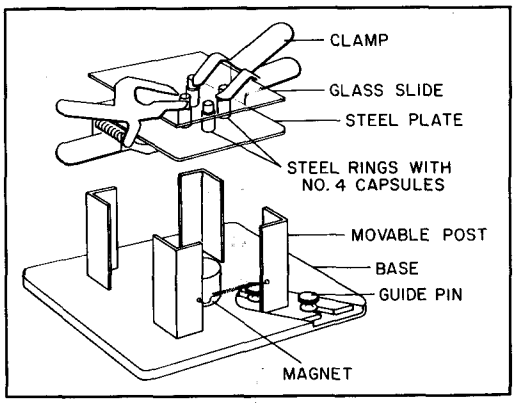

FIGURE 1

Magnetic holder for locating a number of selected single fragments of enucleate cytoplasm or single cells from one culture and embedding them simultaneously. The holder consists of a base plate, circular center magnet, and four right angle posts, one of which is movable, is spring-loaded, and can be locked in position by tightening the knurled nut on the guide pin.

An embedding assembly, held by clamps, is shown raised above the holder. It consists of a steel plate, gelatin capsules set in steel rings, and a glass slide bearing cultured cells on its underside. At this point, the capsules, which contain partially polymerized methacrylate, have been placed in position under selected cells. After a second magnet has been applied to the steel plate to keep the steel rings and capsules in place, the assembly will be inverted.

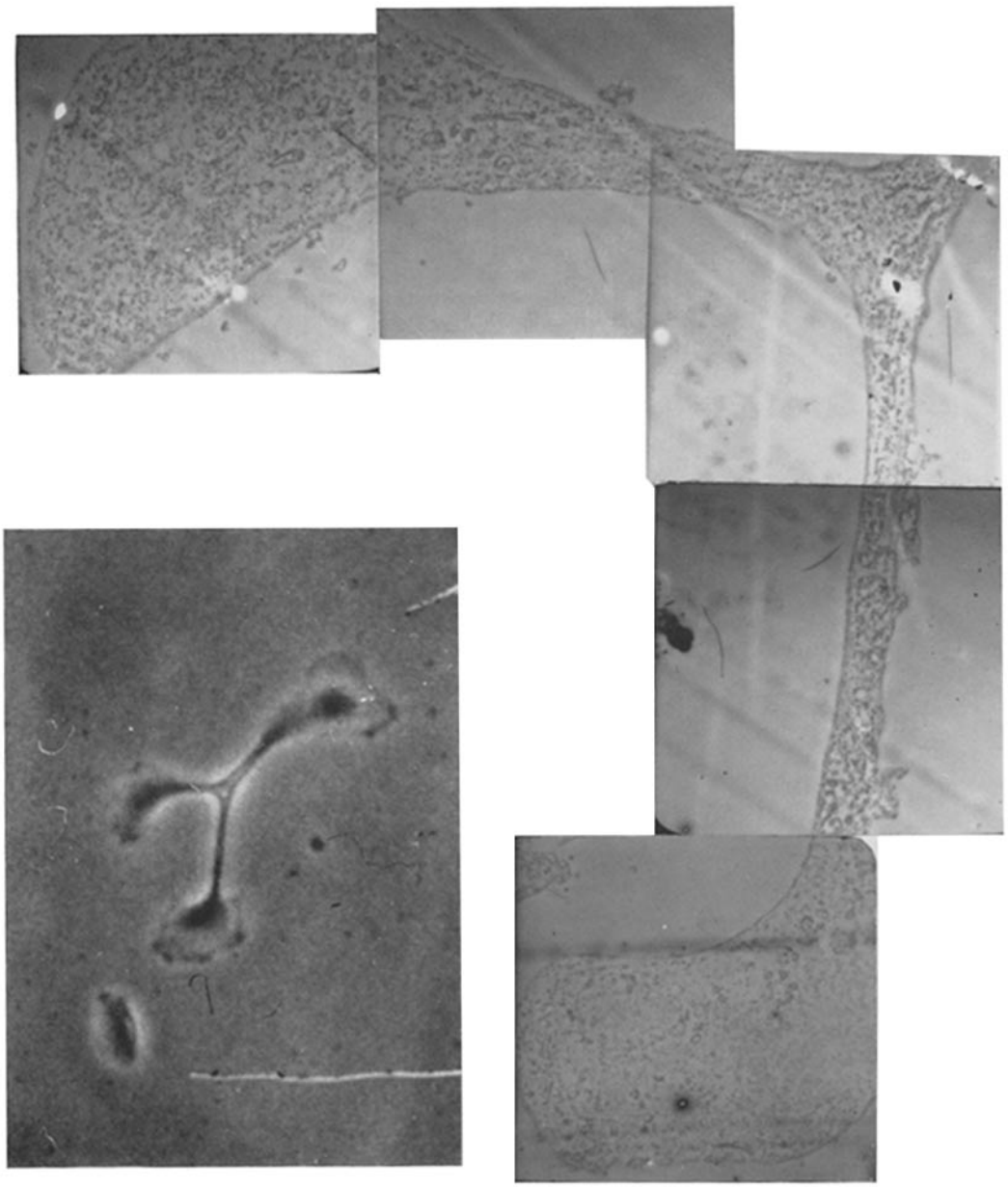

FIGURE 2

An enucleate fragment of an amnion cell (strain A185 21C) in tissue culture produced by micrurgy. Fragment was fixed 11 hours after enucleation. This photomicrograph was chosen to demonstrate positive relocation following embedding and does not exemplify characteristic morphology at this stage. *Lower left:* phase contrast photomicrograph taken just before fixation, × 400. *Top and right:* electron micrograph of a section including the two lower limbs of the cut fragment in the photomicrograph, × 2700.

placed on top of the circular magnet to increase the working area of the magnetic pole face. No. 4 capsules, containing partially polymerized methacrylate, are held in steel rings, 9 mm high, with walls 1 mm thick and an inside diameter providing a push fit. The rings support the capsules and, with the aid of the magnet below, keep them firmly in position. They also permit placement of capsules in close proximity. After the capsules have been placed in position under the slide, the embedding assembly is clamped, removed from the holder, and inverted (see section on Embedding).

Preparation of Cells

Cells suitable for enucleation are produced by planting human amnion cells, strain A185 21C (10), in Rose chambers and treating them as described previously (9). To facilitate removal of capsules after embedding, the bottom coverslip of the chamber is replaced by a microscope slide, 2 by 3 inches, cut to fit the chamber. The slide is coated with a nontoxic 1 per cent solution of Siliclad (Clay-Adams, Inc., New York). Cells adhere less firmly to this surface than to glass, assuring their removal with the methacrylate after embedding.

In selecting cells for microsurgery and for uncut controls, particular attention must be given to their grouping so that no more than two or three are contained in a 5 mm circle corresponding to the diameter of a No. 4 capsule. The groups must be 2 to 3 mm apart and the cells must be spaced to allow separate sectioning of each.

After a cell has been selected and subjected to microsurgery, cells growing in the vicinity are cleared away with the microtool. The cleared area is then circled on the underside of the slide with a diamond marking objective. Photomicrographs are taken during observations of the enucleate fragments and again immediately before fixation. Each photomicrograph is taken at a low magnification to include not only the selected fragment and the cleared area but also a few nearby cells to aid in future orientation and location.

The slide is removed from the Rose chamber and, without being washed, is placed in 1 per cent buffered osmium tetroxide (11) at 4°C for 15 minutes. The lids of staining dishes, 75 by 58 by 30 mm, are convenient in size and depth for fixation and dehydration procedures, and can be covered with the dish itself or enclosed in another vessel.

A standard dehydration schedule is followed. The slide is placed for 15 minutes each in a series of graded alcohols and for 30 minutes each in several changes of absolute alcohol. At some time during the process, the back of the slide is wiped dry and the small circles marking the position of the enucleates are located by reflected light. To facilitate embedding, a larger, more visible circle about the diameter of a No. 4 capsule is drawn with a diamond marking pencil around each of the smaller circles. Two or three small circles can be included in the larger one if their spacing permits. They must be sufficiently far apart so that (*a*) each cell fragment can be isolated at the apex of one of the pyramids formed when the block is trimmed, and (*b*) each of the pyramids on the block can be sectioned separately without interfering with the others.

After dehydration, the slide is pretreated for embedding by immersion for 15 minutes each in equal parts of absolute alcohol and methacrylate, in two changes of methacrylate alone, and in methacrylate with 1 per cent benzoyl peroxide. (A mixture of methyl and butyl methacrylate, 1 part to 9 parts, is used in all steps.) Other embedding media have not been tested but it is presumed that if they fracture from glass they would be suitable.

Embedding

The slide is inverted and clamped in the magnetic holder (Fig. 1), cells down, over gelatin capsules containing partially polymerized methacrylate. With a pencil or other nonmagnetic instrument, and under observation from above, the capsules are moved quickly into position under the areas circled on the slide. The movable post of the holder is loosened by unscrewing the guide pin, allowing the slide to settle directly on the capsules. Since the capsules often vary slightly in height, the glass slide is pressed down firmly by hand. The guide pin is then relocked before proceeding. Two No. 18 ball-joint clamps, adapted to the thickness of the embedding assembly, are inserted simultaneously, one from each side, and clamped in place. The entire unit, consisting of slide, capsules, and steel plate, is then lifted from the holder, placed on another magnet, and inverted. The magnet holds the capsules firmly in place and

keeps the metal rings enclosing them up and out of any methacrylate that may leak out after the assembly is inverted. When a number of units are to be polymerized at one time in a small oven, several can be attached to the oven ceiling by their magnets.

After polymerization, the unit is removed from the oven and disassembled. At this time a line is marked with a wax pencil down the side of each capsule and onto the slide at the point of attachment. The capsules are cracked off the glass by placing the slide on a piece of dry ice (2). Each block is then mounted in a chuck and its relationship to the culture is established with the aid of a binocular microscope. The block is first positioned grossly by aligning the wax pencil marks on slide and capsule, and then more exactly by bringing the small circle marked on the slide into focus. The area on the face of the block which corresponds to the small circle is then located by focusing down. The slide is removed and, with the photomicrographs serving as guide, each fragment is found and identified. The block is trimmed into pyramids, each containing a single enucleate fragment. The pyramids are sectioned with a Porter-Blum microtome and the sections are mounted on electron microscopy grids.

Fig. 2 demonstrates that the identity and orientation of a living enucleate piece photographed prior to fixation can be reproduced in an electron micrograph of a section of the enucleate.

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