A TECHNIQUE FOR THE CULTIVATION AND PREPARATION OF TISSUE CULTURES FOR ELECTRON MICROSCOPY

by

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I. INTRODUCTION

Since the first publication by PORTER, CLAUDE, AND FULHAM (1945)[1] on the electron microscopy of tissue culture cells, interest in this subject has been aroused in several laboratories, and the need for a detailed description of an adequate technique has become apparent. The papers by PORTER, CLAUDE, and their collaborators give only a general outline of their method, and do not contain enough detail to permit easy repetition. We have therefore developed a modification of PORTER and CLAUDE's technique, which we propose to describe in full detail, so as to make it easily reproducible by anyone familiar with the principles of tissue culture technique.

The description falls naturally into two parts; firstly, the tissue culture method and subsequent fixation technique, which provides cells with the required characteristics for electron microscopy, and secondly, the preparation and mounting of the material for electron-microscopical examination. A. M. is responsible for the first part, and S. G. T. for the second.

In order to make full use of the resolving power of the electron microscope, very thin specimens are required. It is necessary, therefore, that tissue cultures should have a zone of growth consisting of only one layer of cells, and that the cells themselves should be thin and well spread out. Every trace of the nutrient culture medium, as well as other debris, must be easily and completely removable, preferably after fixation of the culture. These conditions are fulfilled if cultures are grown in a fluid medium where migration and growth take place only in the two dimensions of the glass surface. The liquid medium used in our method is the exudate of a fowl plasma-embryo extract coagulum. It is capable of maintaining normal, healthy growth for a number of days in a variety of tissues (*e.g.*, adult newt liver, kidney and ovary; embryonic chick gut, sclerotic, osteogenic, and chondrogenic tissue; spleen from young mice; and embryonic human osteogenic tissue).

Briefly, the procedure is as follows: the cultures are grown in the fluid medium mentioned above on a glass coverslip previously coated with a film of polyvinyl formal resin ("formvar"). They are then fixed and washed, the explant is removed, and the film with the adherent cells is stripped from the glass and mounted on a fine copper or

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stainless steel grid ready for examination in the electron microscope. Each step in this procedure will be fully discussed.

II. PREPARATION OF COVERSLIPS

1. *Cleansing*

New glass coverslips 1.25 inches square are used. For thorough cleansing, a dozen or so are placed in a stainless steel rack in such a way that they cannot touch one another. The rack is then lowered into a beaker of "Dreft"* solution and boiled. After being boiled for some minutes, the coverslips, still in the racks, are rinsed in several changes of distilled water. Finally they are rinsed in absolute alcohol, and after being drained are passed through a flame to ignite the remaining alcohol. They are then clean, dry, and ready for coating with formvar.

2. *Coating with formvar*

The formvar film is applied by dipping the coverslips individually in a suitable solution of polyvinyl formal in either dioxane, ethylene dichloride or chloroform. Most of our work has been done with a solution of 0.7 g of polyvinyl formal in 100 ml of dioxane (commercial), but in specifying the concentration of the solution it must be borne in mind that this may depend to some extent upon the particular specimen of polyvinyl formal used, and also upon the way in which the coverslip is withdrawn from the solution, as this affects the draining of the surplus liquid. We find that it is essential to allow the coated coverslips to dry in a vacuum desiccator as otherwise the film acquires a conspicuous bloom and is useless for electron microscopy. The solution in dioxane appears to be the worst offender in this respect, but the other solutions are also liable to give trouble unless the vacuum desiccator is used.

In full detail, the coating process is carried out as follows: A clean, dry coverslip is held in a small metal paper clip and dipped vertically into the formvar solution contained in a wide necked reagent bottle which is about half full. Care should be taken not to dip the clip into the solution. It is then slowly and steadily withdrawn from the liquid, the withdrawal occupying five or six seconds, and transferred as rapidly as possible to the vacuum desiccator where it hangs vertically by the hooked paper clip from a suitable frame while drying *in vacuo*. Any coverslips which dry with a frosted appearance must be discarded. If ethylene dichloride or chloroform solutions are used it is worth attempting the preparation without the vacuum desiccator, but if the bloom appears, the technique described above is essential.

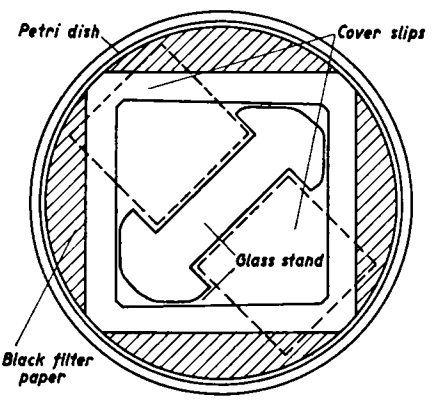

Fig. 1. Petri dish with coverslip stand

3. *Sterilization*

The formvar coated coverslips are placed in sterile Petri dishes containing glass holders (Fig. 1) as used in the *Strangeways Laboratory, Cambridge*. They are then sterilized by exposure for ten to fifteen minutes to the

* Dreft is the trade name of a soapless detergent which is very satisfactory for cleaning glassware.

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short-wave ultraviolet radiation from a 125 watt mercury discharge lamp. After sterilization the lids are replaced on the dishes and the coverslips remain in the stands throughout the subsequent manipulations. The coverslips should be sterilized at least one to two hours before use as they take some time to cool.

III. TISSUE CULTURE TECHNIQUE

1. *Preparation of plasma exudate ('serum')*

Fowl plasma and chick embryo extract are usually mixed in a proportion of 1 : 1, though this may be varied for different tissues, and allowed to coagulate in a small glass tube at +37° C. The clot is then minced with a sharp cataract knife, but the pieces should not be cut too small. After incubation for not less than 30 minutes at +37° C, the first exudate may be pipetted off and stored in a sterile tube ready for use. The remainder of the clot is then cut into still smaller pieces, and after renewed incubation a second supply of fluid can be obtained.

2. *Preparation of ring clots*

Ordinary hanging-drop cultures in fluid media have several disadvantages, such as the tendency of the drop to run to the edge of the coverslip when the culture is accidentally tilted, and the necessity of incubating the cultures "upside-down" until the explant adheres to the coverslip. These drawbacks can be avoided by spreading the fluid medium thinly within a circular wall of a coagulated mixture of fowl plasma and chick embryo extract ("ring clot"). A further advantage of the ring clot is that it exudes serum during incubation and thus supplies the culture with additional nutrient material. In preparing the ring clots great care must be taken to avoid damage to the fragile formvar film.

The following procedure is relatively safe. Small drops of plasma, alternating with drops of embryo extract, are placed on the formvar-coated coverslip to form a circle of approximately 1 cm diameter. They are then made to fuse to form a continuous ring. This may be effected with a cataract knife held very flat, or with a fine glass rod, the end of which should be well rounded and smooth. Even if great care is taken, however, it is not always possible to avoid touching the formvar film with the instrument when proceeding in the manner described. For this reason it is safer to bring about fusion of the drops by gently blowing with a pipette. If the resulting ring appears too thick, the pipette can be used to remove the excess before coagulation begins.

It is very important that coagulation of the ring clot should be complete before serum and explant are transferred to the coverslip. If this precaution is disregarded, the still uncoagulated part of the ring clot will mix with the serum and form a film of coagulated plasma which later seriously interferes with electron-microscopical observation. It is advisable to prepare the ring clots before cutting and washing the explants to make sure that they have coagulated by the time the explants are ready.

3. *Transferring the explants to the coverslips*

Usually, cultures in their first passage are subcultivated on to the formvar-coated coverslips, but such material as embryonic chick gut or chondrogenic tissue from an early embryo, may be explanted on to the formvar immediately after dissection.

After being washed in a balanced salt solution, *e.g.*, TYRODE's solution, the explants

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are placed in a depression slide or flat bottomed watch glass in a sterile petri dish and covered with serum. Each explant is then transferred to the centre of a coverslip in the space surrounded by the ring clot. They should be allowed to drop from the pipette, together with the serum, and on no account should the pipette touch the formvar film. More serum is now added, until the area surrounding the explant is covered completely. The excess is then carefully removed with a fine pipette or the cut edge of a strip of sterile filter paper. This can be effected by tilting the petri dish containing the two coverslips on their stand, so that the liquid accumulates where it can be sucked off by applying the pipette or filter paper to the edge of the ring clot. Only a very thin film of serum should remain. If this manipulation has been carried out successfully, so that no excess of fluid is present, the explants will adhere firmly to the coverslips. There is no need to incubate such cultures "upside down".

IV. PREPARATION OF CULTURES FOR EXAMINATION IN THE ELECTRON MICROSCOPE

1. *Fixation*

The choice of a fixation technique was determined by the following considerations.

Exposure to room temperature after incubation, rinsing with (warm) saline, and, above all, slow fixation, may cause retraction of the tissue culture cells and the formation of artefacts such as coarse granulation of the cytoplasm, vacuolization, shrinkage of the nucleus, and changes in the appearance of the mitochondria. It is necessary, therefore, that cultures should be fixed within a few seconds of being taken out of the incubator, and that rinsing before fixation should be omitted. If cultures are not washed before fixation it is not always possible to remove every trace of the serum, and with most fixing agents this residue forms a coarse, insoluble precipitate which renders the cultures useless for electron microscopy. By using osmium tetroxide in solution this difficulty can be overcome, since the precipitate formed can be removed by subsequent washing.

We have obtained well fixed cells, with negligible shrinkage and retraction, by adopting the following procedure:-

The coverslip is loosened from the depression slide to which it was sealed, lifted, and the serum is carefully removed by applying a cut strip of filter paper to the edge of the ring clot. The culture is now covered with a drop of a 2% solution of osmium tetroxide, inverted over a watch glass to prevent debris from accumulating on the formvar film, and fixed for 30 seconds. It is then washed for about 3 hours in 3–4 changes of distilled water. From time to time the jar in which the coverslips are being washed should be gently agitated.

2. *Storing*

Whenever possible the subsequent stages in the preparation of cultures for electron microscopy should follow immediately upon fixation and washing. Where this is not feasible, the cultures must be stored in a suitable preserving fluid. For this purpose 70% alcohol proved useless as it causes the formvar film to adhere firmly to the coverslip, so that the film cannot be removed without damage to the cells. We have found formaldehyde in an 8%–10% solution very satisfactory. It is effective as a preservative, damages neither the cells nor the formvar film, and has no influence on the subsequent manipulations. The only precaution that has to be observed when formaldehyde is used

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is to remove the explant before the cultures are transferred to the preservative. This is important, as the explant is hardened by storage in formalin, and its removal at a later stage may cause damage to the outgrowth.

3. *Stripping and mounting the cultures*

A formvar film, on which tissue culture cells have been grown, cannot be easily removed from the glass; when the coverslip is slowly immersed in water the film will not float off as does a freshly prepared formvar film. The procedure for detaching the film and mounting a selected part of it on the copper grid, which is the usual type of object support for electron microscopy, consists of the following steps:

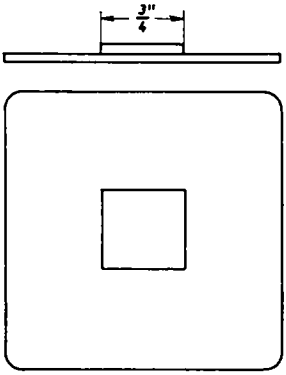

Fig. 2. Glass or perspex platform

a. After a final rinse in distilled water the coverslip is placed with the cells upward on a glass or perspex platform (shown in Fig. 2) on the stage of a binocular dissecting microscope and illuminated from below; the binocular microscope is used at a magnification of about 15 diameters throughout. The original tissue explant is now removed with a fine needle, if it has not already become detached during the washing. It is sometimes found that a film of plasma has formed over the growth, but this is often removed with the explant.

b. Next, the formvar film is cut along three sides of a rectangle 3–5 mm wide, in the centre of which are the cells which, under the light microscope, appear to be suitable for electron microscopy. The cuts (Fig. 3) are made with a small knife consisting of a piece of safety razor blade, 3 or 4 millimetres wide, held in a suitable handle. If the outgrowth of cells is sufficient, two rectangles can be marked out by making one cut through the centre of the culture so that two preparations may be obtained from one culture. If the outgrowth is less extensive all the cells will lie in the centre of one rectangle.

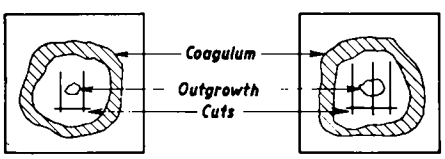

Fig. 3. Cutting the formvar film

c. The coverslip is now immersed in distilled water in a glass dish about 2 cm deep standing on the stage of the binocular microscope, and held at about 45 degrees to the horizontal (Fig. 4). The cells are on the upper surface, with the narrow edge of the rectangle horizontal and below the group of cells. With a fine needle the film is now lifted from the glass along the horizontal cut until a small flap of film floats free of the glass. Next, with a pair of carefully ground square-ended forceps, this piece of film is seized and very gently pulled

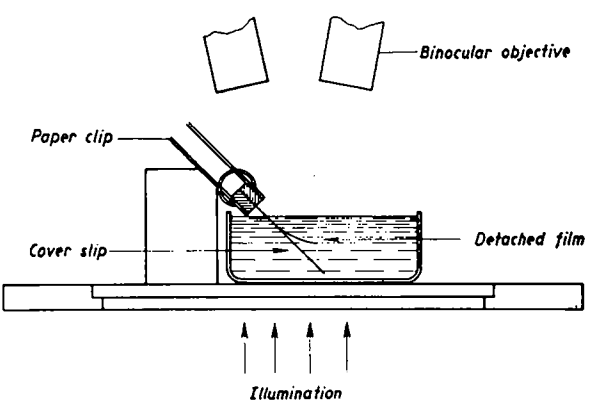

Fig. 4. Detaching the film

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upwards until a strip of film about 5–7 mm long carrying the selected cells floats free of the glass, though still attached at its upper end (Fig. 4). The coverslip is then carefully withdrawn from the water and placed on the previously mentioned platform under the dissecting microscope. The fourth side of the rectangle is now cut through; when doing this it is desirable to have a needle ready to hand in case the film sticks to the knife. A small piece of film carrying the cells is now free of the glass and ready for mounting on a copper grid.

d. On the stage of an ordinary microscope with a two-thirds objective is placed a glass dish about 1–5 cm deep filled with distilled water. In the water is placed a stainless steel block drilled with a hole a little less in diameter than a copper specimen grid, one of which rests on top of the block just below the water surface (Fig. 5). Also attached to the microscope stage is a siphon by means of which the glass dish may be very slowly emptied. The dish is placed so that the copper grid is central in the field of view of the microscope. These preparations should be made before stage three of the process is reached, for if the coverslip with the detached piece of film is allowed to dry the film will again adhere firmly to the glass.

The next step is gradually to immerse the coverslip in the water at an angle of about 45 degrees until the detached piece of film floats freely on the water surface. It should be held with a needle as the coverslip is withdrawn, and then manoeuvred until it is above the grid. A metal bridge with a glass tube about 1 cm in diameter projecting downwards is now placed in position so that the floating film is within the glass tube which dips into the water. The bridge is held by the jaws of the mechanical stage (Fig. 5). With the stage controls, the piece of film can now be moved about on the water surface while it is viewed through the microscope, and the most suitable cells brought above the centre of the copper grid. The water is then slowly siphoned out of the dish and the film allowed to settle on to the grid with the required cells in the centre. This latter technique was first described by SMILES[5].

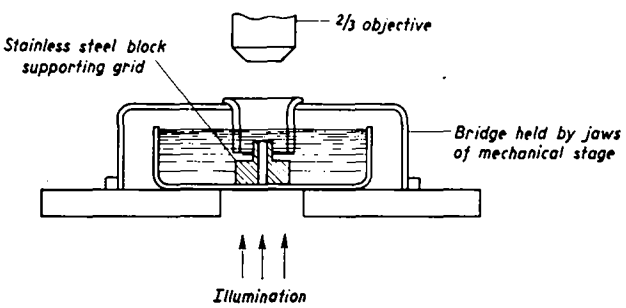

Fig. 5. Mounting the chosen cells

The stainless steel grid support is removed from the water, drained with filter paper and put to dry in a desiccator, after which the specimen is ready for the electron microscope.

It is found that sometimes the films do not adhere securely to the grid after drying, a difficulty which can be obviated by making the grids adhesive before use by immersing them in a 1% solution of polyisobutylene. This appears to be a useful measure provided care is taken not to clog the grid openings with the adhesive.

V. THE INTERPRETATION OF ELECTRON MICROGRAPHS

The general appearance of the tissue culture cells pictured in Fig. 6–9 differs little from that of material fixed and stained according to one of the conventional cytological

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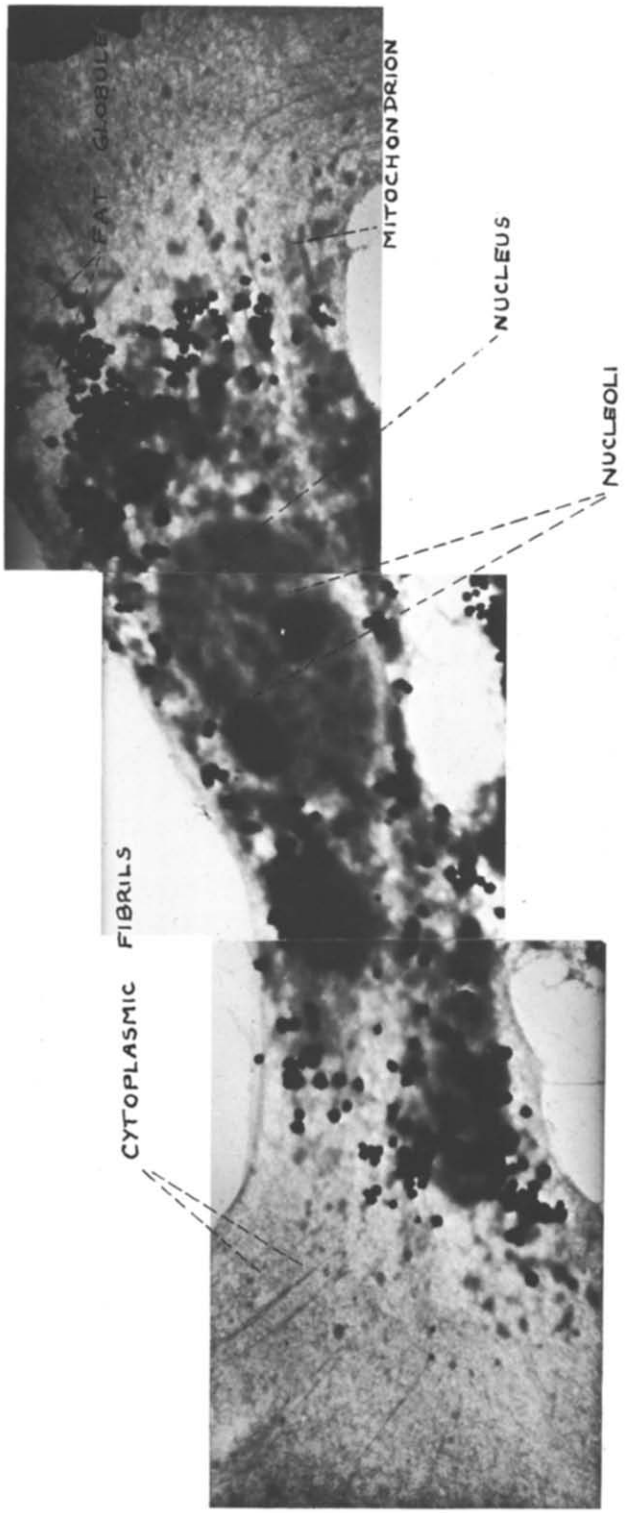

Fig. 6. Connective cell from a culture of young mouse spleen. Note nucleus with two nucleoli, fat globules, mitochondria, reticular background structure of the cytoplasm, and cytoplasmic filaments. Taken at × 5000 and 52 kV.

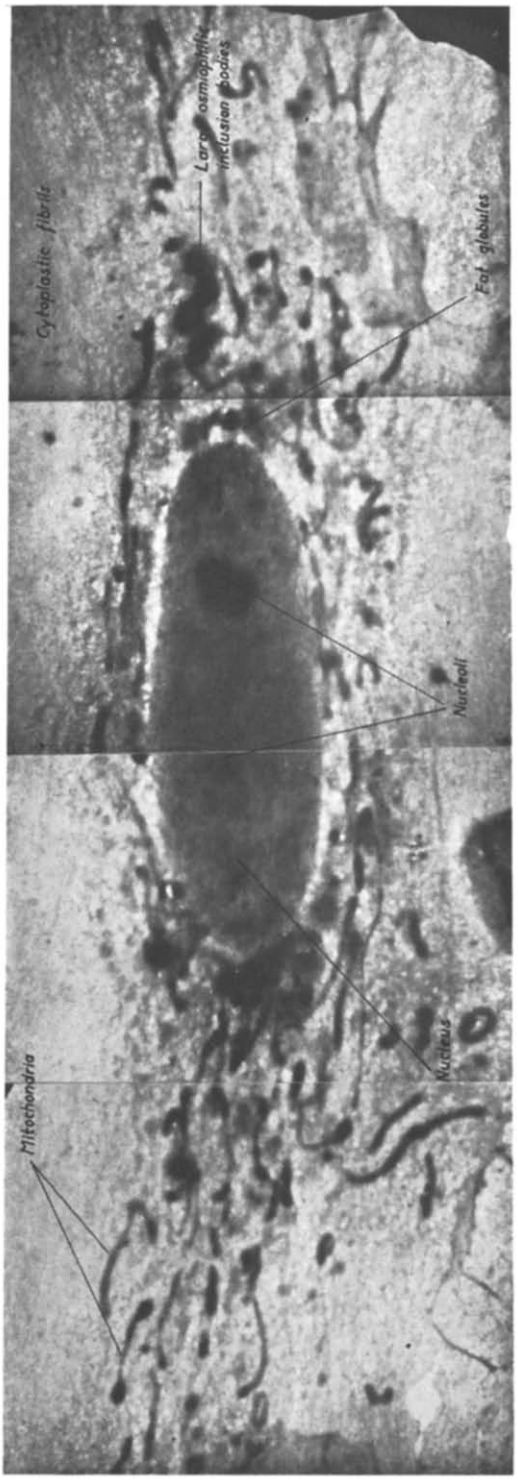

Fig. 7. Epithelial cell from a culture of embryonic chick gut. Note nucleus, filamentous mitochondria, large osmiophilic bodies (probably identical with those described as Golgi bodies by PORTER *et al.*), cytoplasmic ground structure, and cytoplasmic fibrils. Taken at × 5000 and 90 kV.

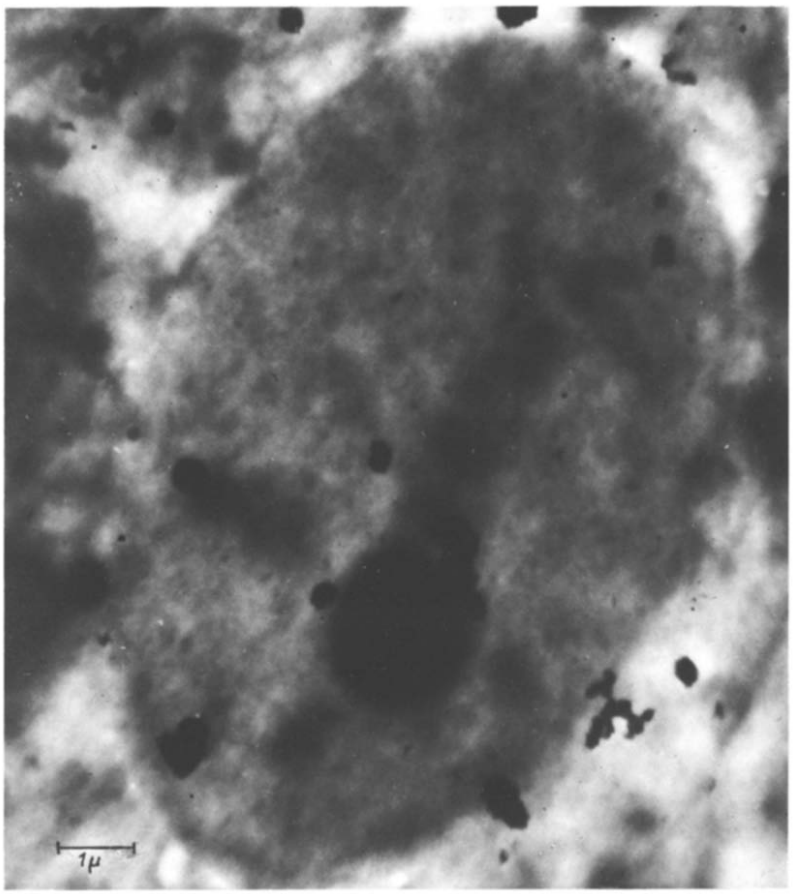

Fig. 8. Nucleus of epithelial cell as in Fig. 7. Filamentous structure resolved in the area of the nucleus probably partly due to superimposed cytoplasmic structure. Taken at × 5000 and 52 kV.

procedures. They are sufficiently thin to permit electron microscopy of the nuclear region, and structural detail is revealed in the area occupied by the nucleus, as well as in the surrounding cytoplasm. The identification and interpretation of the fine structure is difficult because cell components situated at different levels appear in focus simultaneously, owing to the great depth of focus of the electron microscope objective, so that it is often impossible to ascertain their true position in the cell. The physiological condition of the cell at the time of fixation, as well as fixation technique and subsequent treatment, influence the final appearance of nucleus and cytoplasm as revealed by the electron microscope.

Electron micrographs of osmium tetroxide-fixed tissue culture cells published by PORTER, CLAUDE, AND FULLAM (1945)[1], CLAUDE, PORTER, AND PICKELS (1947)[2] and PORTER AND THOMPSON (1947 and 1948)[3,4] picture the cytoplasm as a network, the mesh size of which varies according to the type of cell used in each investigation. In cells of embryonic chick gut (PORTER *et al.*, 1945)[1] this reticulum is very coarse and superimposed upon a fine granular background. Our Fig. 6, 7, and 9 also show a cytoplasmic network which differs from the structures demonstrated by the authors mentioned

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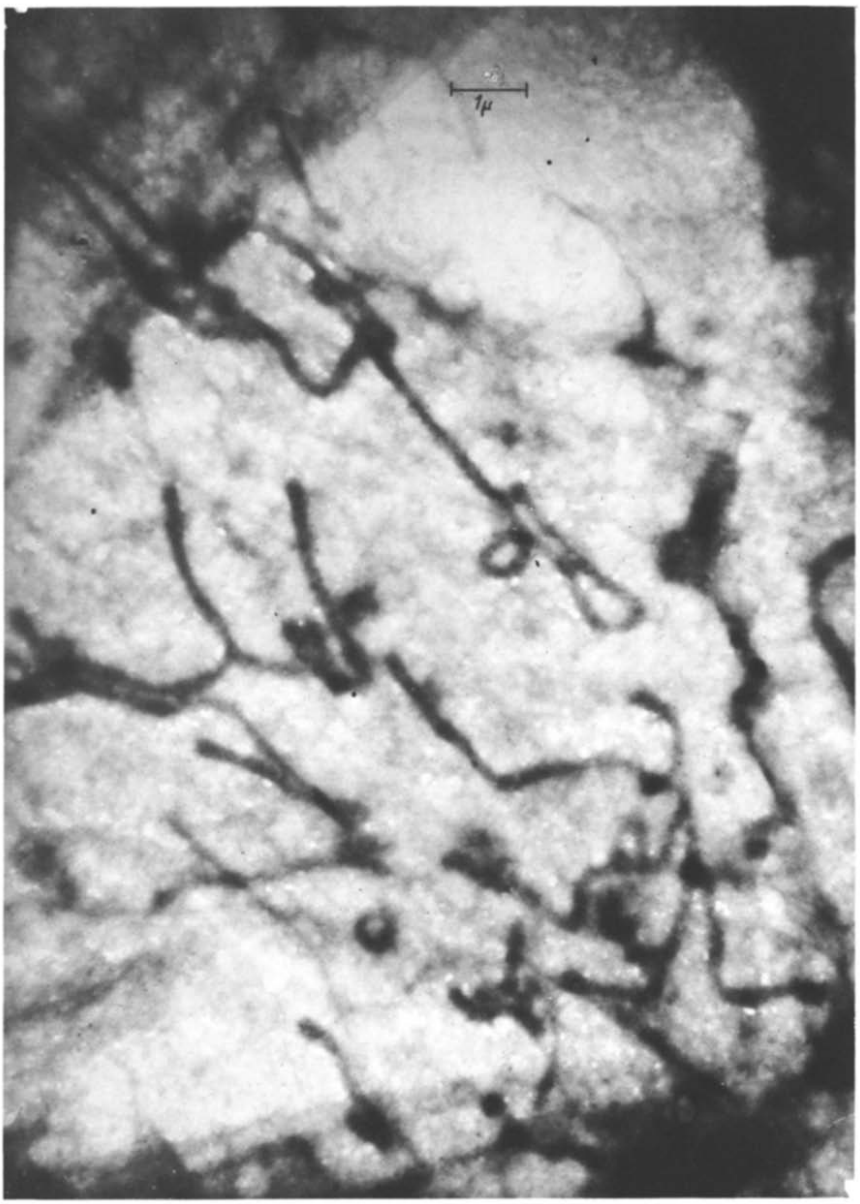

Fig. 9. Filamentous mitochondria in epithelial cell as in Fig. 7. Note correlation between areas of greater density in the mitochondria and the presence of underlying cytoplasmic reticulum. Taken at × 5000 and 90 kV.

above mainly in its finer mesh. It seems possible that this fine background reticulum may represent the same cytoplasmic component as the granular ground substance figured by PORTER *et al.* (1945)[1] after osmium tetroxide fixation of up to 24 hours. These authors noted that the granular appearance of the cytoplasm becomes more pronounced with prolonged fixation; the fact that we allow the osmium tetroxide to

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act upon the cells for only 30 seconds may therefore account for the difference in cytoplasmic background structure. There is an indication of a coarser cytoplasmic network in our micrographs, mainly in the vicinity of the cell nucleus (Fig. 6, 7, 8); we have, however, no evidence that this corresponds with reticular structures mentioned in the publications cited above.

Other conspicuous features of the cytoplasm are filaments or fibrils of varying width and length. In the mouse spleen cell (Fig. 6), long and rather dense filamentous structures may be observed mainly in the relatively clear cytoplasmic regions at either 'end' of the cell, while a system of long, thin fibrils is present in the cells of a chick gut culture (Fig. 7). The latter resemble the fibrils seen by BANG AND GEY (1948)[6] in electron micrographs of rat fibroblasts. No information is yet available as to the possible correlation between these structures and components of the living cell.

The filamentous mitochondria pictured in Fig. 9 exhibit marked variations in density as previously described in the same type of cell by PORTER *et al.* (1945)[1]. Close examination of the micrograph reproduced in Fig. 9 shows these variations to be due to the presence of cytoplasmic structures underneath or above the filaments. In our material, there is no evidence of internal structure in the mitochondria.

The identification of structural detail apparently present in the nuclei of our tissue culture cells must be approached with great caution. As already mentioned, the depth of focus makes it very difficult to determine the exact position of cell components in electron micrographs, and 'intranuclear' filamentous structures such as those shown in Fig. 8 may well be part of the cytoplasmic layer covering the nucleus. Fig. 6 shows clearly how the cytoplasmic pattern with numerous inclusion bodies can be superimposed upon the picture of the nucleus itself. It will be possible to recognise true intranuclear structure only when a suitable nuclear staining technique for electron microscopy has been devised.

It is to be hoped that the development of cytochemical tests, comparable to those in use in light microscopy, will help to overcome the difficulties which at present limit the usefulness of electron microscopy in the study of tissue culture cells.

ACKNOWLEDGEMENTS

We wish to express our thanks to Professor J. T. RANDALL, F. R. S., for his interest and encouragement, and to acknowledge our indebtedness to Dr H. B. FELL for her valuable advice and guidance.

SUMMARY

A technique for providing thin tissue culture cells for electron microscopy is described in detail. The difficulties encountered in interpreting electron micrographs of such cells are discussed.

RÉSUMÉ

Les auteurs décrivent en détails une technique pour l'obtention de cellules minces dans les cultures de tissus étudiées en microscopie électronique. On discute les difficultés rencontrées lors de l'interprétation de micrographies électroniques de telles cellules.

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ZUSAMMENFASSUNG

Eine Arbeitstechnik zur Gewinnung dünner Gewebekultur-Zellen für die Elektronenmikroskopie wird eingehend beschrieben. Die Schwierigkeiten, denen man bei der Interpretation von Elektronenmikrographien solcher Zellen begegnet, werden auseinandergesetzt.

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