

# A Cellophane-Strip Technique for Culturing Tissue in Multipurpose Culture Chambers\*

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## ABSTRACT

A new technique for the cultivation of living tissues in the multipurpose culture chamber is described. This procedure employs strips of cellophane as the agent for anchoring tissue explants to the coverslip walls of the chamber and disposes of the time-honored plasma-clot technique. The primary advance embodied in this procedure lies in the fact that cells emigrating from so-cultured explants manifest themselves in a highly differentiated manner comparable to the cells of origin, whereas the outgrowth from the same types of tissue in plasma clots results in a more undifferentiated type of growth. Comparisons of outgrowths from embryonic thyroid, bone, and muscle (chicken) are photographically documented, and attention is called to certain cytochemical methods which further corroborate the differentiated quality obtained with the cellophane-strip technique.

## INTRODUCTION

After a prolonged investigation of thyroid gland fragments of chick embryo (12, 13) which were cultured in clotted cockerel plasma on coverslips of multipurpose culture chambers (5-16), it was decided to attempt their cultivation by some method which would eliminate the plasma variable. We had already tried to initiate cultures by merely allowing free thyroid gland fragments to gravitate to the coverslips. This procedure resulted in a rather poor outgrowth and generally was ineffective in establishing cultures. We had cut round pieces of perforated cellophane (1), placed them in multipurpose culture chambers, and allowed the surface tension and gravitational

pull to anchor the fragments. This attempt too, was highly unsatisfactory and again a very poor outgrowth resulted. In addition, the perforations in the cellophane did not permit ideal optical conditions and also influenced the direction of outgrowth of the few explants which were cultivated. With the hope that the cellophane had more to offer, strips were cut from Visking dialysis tubing, and by the methods described it was found that they were most satisfactory agents for anchoring explants to the glass. Results were now superior to any chamber method previously used. Moreover, we found that the cultures developing in the chambers (in a relatively flat plane for microscopic observation) were composed of a mixture of highly differentiated cells with characteristics strikingly similar to their histological counterparts. Since differentiating features were also observed in explants of embryo chick muscles (with C.M.P.) and bone (with T.O.S.) (14) it was felt that the implication of this technical procedure to tissue culture technique warranted a formal notation.

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### Materials and Methods

*Standard-Size Multipurpose Culture Chambers (2 x 3 inches).*—In assembling the chambers, a flamed coverslip is laid upon a retaining plate wiped with alcohol. Four fragments of tissue (approximately 1 mm. square) are then placed radially about half a centimeter from the center of the coverslip. Two strips of cellophane ( $\frac{1}{4}$  x 4 inches) cut from Visking dialysis tubing ( $1\frac{1}{8}$  inch diameter) and prepared as indicated below, are laid across the explants (Fig. 1). These explants have just enough nutrient overlying them so that air bubbles are not trapped between the glass and the cellophane. The rubber gasket is next positioned, and the chamber closed with another coverslip and retaining plate. After the four screws are gently tightened, the pieces of cellophane tape may be pulled so that wrinkles are smoothed. The explants are then securely lodged against the coverslip wall. The technique is completed by tightening the screws with the gasket-penetrating air vent needle in place and filling the chamber with the desired nutrient fluid (Fig. 2). Rather than using two  $\frac{1}{4}$ -inch wide strips of cellophane, a single  $\frac{3}{4}$ -inch strip may be employed. The broader widths require extra care as air bubbles are more easily trapped. In addition, if there is too much fluid on the coverslip, the explants may be moved to one side as the cellophane is positioned.

*Super-Size Multipurpose Culture Chambers (4 x 4 inches).*—These larger chambers have a center aperture which is  $2\frac{1}{4}$  inches in diameter and require 3 x 3 inch No. 1 or No. 2 coverslips. They can be used with  $\frac{1}{4}$  inch and  $\frac{3}{8}$  inch thick gaskets and thus yield two chamber sizes. One contains approximately 16 ml. and the other 27 ml. of nutrient in contrast to the standard size multipurpose culture chambers which contain only 2 ml. of nutrient. The  $1\frac{1}{8}$  inch dialysis tubing may be used, cut into multiple strips  $\frac{1}{2}$  to  $\frac{3}{4}$  inches in width and 5 to 6 inches in length. A somewhat better technique has been developed using strips 2 x 6 inches cut from a heavier grade of Visking dialysis tubing ( $1\frac{1}{8}$  inch diameter). This heavier cellophane is quite easily positioned over a dozen or more fragments without allowing air bubbles to lodge in the interspace (Fig. 3). These large chambers are similarly closed after the gaskets and second coverslips have been positioned (Fig. 4).

*Cellophane Preparation.*—After the cellophane strips have been cut to the desired size, they are immersed in pure ethanol (95 per cent) for a few minutes. Each cellophane strip is then individually removed from the alcohol with sterile forceps and squirted with a fine stream of nutrient such as medium No. 1066 (Connaught Medical Research Laboratories, Toronto, Canada), a variation of medium No. 858 (4). Next they are placed in a sterile petri dish which contains a small amount of medium No. 1066. As the cellophane strips are required for the construction of the culture chambers, they are transferred individually with sterile forceps.

*Nutrient Medium.*—The fluid nutrient used for all of these experiments was composed of Parker's medium No. 1066 (75 per cent), calf serum (20 per cent), and whole egg ultrafiltrate (5 per cent).

### RESULTS

The three tissues which have been examined in culture chambers were derived from skeletal muscle, long bones, and thyroid glands of chick embryos. These examinations were made on cultures of 14-day embryo chicks.

*Muscle.*—Striated muscle fragments which were chamber cultured on coverslips in plasma clots produced an outgrowth of spindle-like cells (Fig. 5). Cross-striated and contracting muscle straps were not observed until similar explants were cultivated by the cellophane-strip technique. Muscle straps then grew radially from the explants and crossed through spindle-shaped and epithelioid cells (Fig. 6). Within a few days, some of these muscle straps developed cross-striations (Fig. 11). Rhythmic contractions were frequent.

*Bone.*—Mid-third fragments of leg bone were prepared according to the endosteal technique of Fell (2) by scraping off the periosteum and removing the marrow. In plasma clot cultures the proliferation resembled that obtained with cultured muscle explants (Fig. 7); however, after prolonged cultivation (2 to 4 weeks), cells giving an alkaline phosphatase reaction (3) were found near the explants. The wide and multilayered sheet of fibroblasts made it impossible to ascertain which cells were producing alkaline phosphatase. With cellophane-strip techniques, the bone fragments produced an outgrowth of fibroblastoid cells, but after 5 or 6 days another type of cell appeared close to the explants which was darker (B&L, phase contrast) than the fibroblastoid elements (Fig. 8). These did not have the fat droplets within their cytoplasm which were typical of the fibroblastoid cells, but did have a large number of dark cytoplasmic granules. They emigrated very slowly, but were not overgrown by the fibroblastoids. After several weeks of cultivation they were observed in the outgrowth of a large percentage of the explants, and it was established with the calcium cobalt method of Gomori (3) that the cytoplasmic granules of these phase-contrast-dark cells were sites of alkaline phosphatase activity (Fig. 12).

*Thyroid.*—Thyroid gland explants in plasma clots revealed a condition favorable for the proliferation of epithelium. Frequently after 5 days

there was an overgrowth by the stromal elements which became a barrier for successful epithelial emigration (Fig. 9). Usually the epithelium was barely apparent because the fibroblastoid or stromal type of outgrowth was so predominant. Removal of the serum from the nutrient caused the epithelium to proliferate more abundantly, and there was a concomitant decrease in fibroblastoid cells. When I-131 was introduced into the nutrient, it was observed by autoradiographic techniques (13) to be localized in the cytoplasmic granules of the epithelial cells. Thyroid explants cultivated by the cellophane-strip techniques produced broad epithelial sheets (Fig. 10) and contained more numerous and larger cytoplasmic droplets as well as the I-131 positive granules (Fig. 13). These cells developed with or without serum in the nutrient, but in either case the epithelium was more abundant and the fibroblastoid cells less conspicuous than with the plasma-clot technique.

#### DISCUSSION

The results of these studies on three chick embryo tissues (thyroid, bone, and muscle) have shown us that cell differentiation can be intensified, maintained, and observed throughout cultivation in multipurpose culture chambers. Conversely, they also revealed that maintained differentiation could be lost so that dedifferentiation would follow. This was particularly evident in cultures in which muscle straps simply dedifferentiated as they migrated outward from under the cellophane (Fig. 14). Cell differentiation has long been a source of argument against tissue culture analysis of normal cells. Investigators have often felt that many or all emigrations were composed of dedifferentiated or undifferentiated cells. In these studies the cellophane strips seemed to have constrained certain cell types and thus prevented their dedifferentiation. Certainly cells which have the differentiated characteristics of their histological counterparts should permit less equivocal and more substantial data from microscopic analysis by phase contrast, interference contrast, time-lapse cinematographic, cytochemical, and autoradiographic techniques.

Specific advantages of the cellophane-strip technique over plasma clot procedures are as follows:

1. The cellophane affords a protection against fluid currents when exchanging the nutrients.

This is particularly true in cultures which have a lytic action on the clot (thyroid).

2. Plasma clots frequently become loose, contract, roll, and therefore, destroy growth patterns and even entire cultures (muscle). This does not occur with the cellophane.

3. Plasma sources are variable with respect to their clotting as well as to their stimulating and inhibiting qualities. Cellophane strips obviate this variable.

4. Microscopic observations are crisper throughout because of the flatness of the growth field and the uniform thinness of the cellophane. Phase contrast and interference contrast observations, therefore, have a greater range of usefulness.

5. Fixation may be carried out in the chambers which are then opened and the cellophane removed. The coverslips with the explants and all of the cellular outgrowth are now free of substrate optical and biochemical interference and thus are more readily available for appropriate cytochemical techniques.

An additional study with regard to differentiation in normal and malignant tissue is in progress.

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## EXPLANATION OF PLATES

*Abbreviations Used in Figures*

<i>RP</i> , retaining plates.	<i>Ep</i> , epithelial cells.
<i>Cel</i> , cellophane strips.	<i>CM</i> , cell membrane.
<i>cs</i> , coverslip.	<i>M</i> , filamentous mitochondria.
<i>Gk</i> , pure latex rubber gasket.	<i>Alk</i> , alkaline phosphatase-positive granules.
<i>AV</i> , air vent needle.	<i>N</i> , nucleus.
<i>Ex</i> , explant.	<i>gg</i> , dark phase contrast green granules—sites of I-131 uptake.
<i>Fb</i> , fibroblastoid cells.	<i>CD</i> , cytoplasmic droplets.
<i>Ft</i> , fat droplets.	<i>Cel Ed</i> , cellophane edge.
<i>MS</i> , muscle straps.	
<i>Os</i> , osteoblasts.	

## PLATE 384

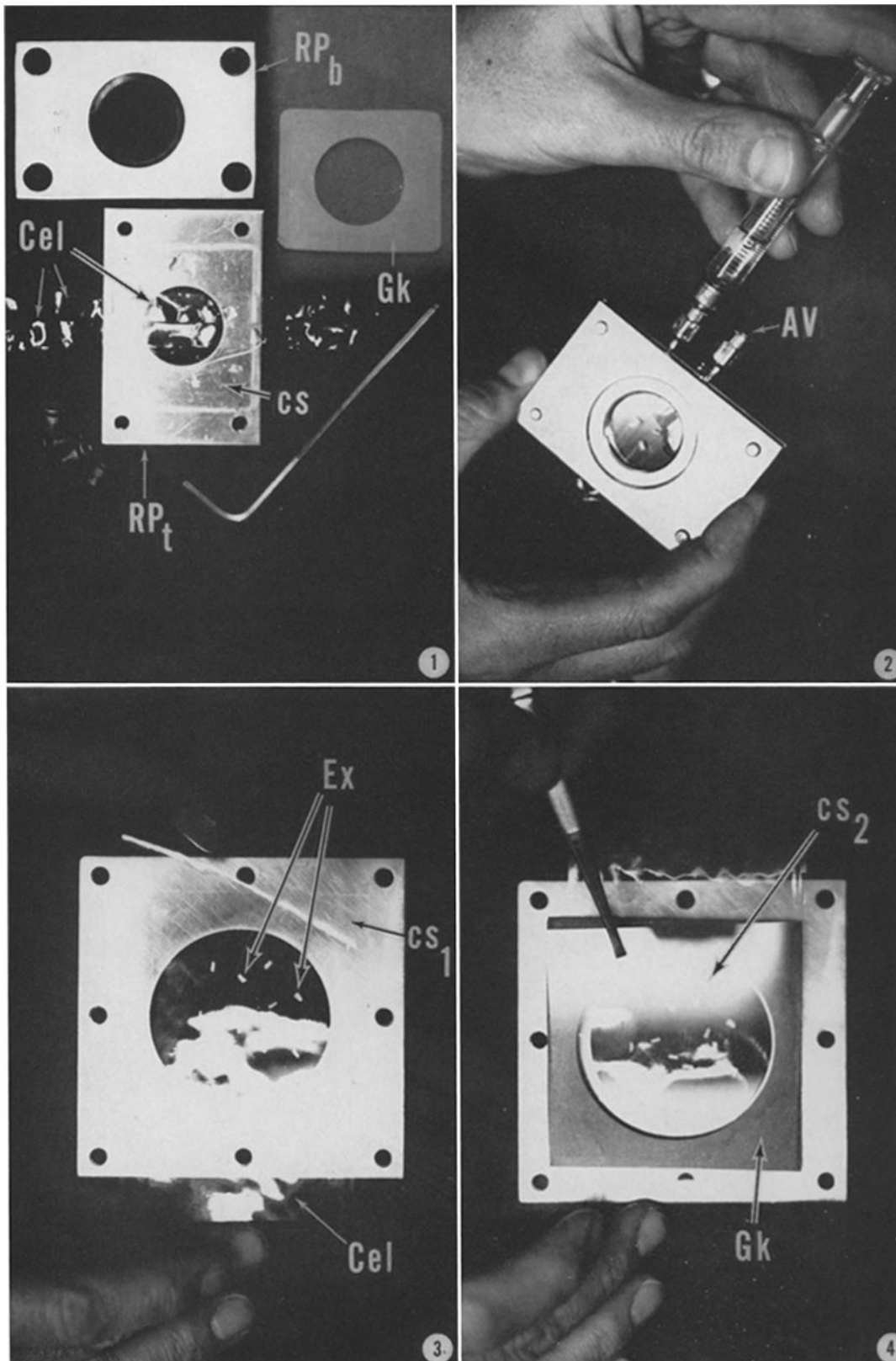
FIGS. 1 to 4. These figures illustrate the technical procedure used with the multipurpose tissue culture chambers of both sizes.

FIG. 1. This figure shows the standard size (2 x 3 inch) multipurpose culture chamber separated to demonstrate all of the component parts. *RP<sub>b</sub>* is the unthreaded bottom retaining plate, *Gk* the rubber gasket, and *RP<sub>t</sub>* the top retaining plate which in the photograph has one coverslip (*cs*) resting upon its surface and two strips of cellophane tape (*Cel*) laying across explants. The Allen-headed screws and the Allen wrench are also shown.

FIG. 2. This photograph demonstrates the technique used for filling each of the chambers after they have been assembled. The second needle (*AV*) without the attached syringe is a vent to allow the escape of the 2 cc. of air in the chamber when the nutrient fluid is injected.

FIG. 3. Here a large size (4 x 4) multipurpose culture chamber is shown as the larger piece of cellophane tape (*Cel*) is laid across explants (*Ex*) on the surface of the 3 x 3 inch square coverslip (*cs<sub>1</sub>*).

FIG. 4. The figure illustrates the placement of the second coverslip (*cs<sub>2</sub>*) for the large size multipurpose culture chamber on top of the rubber gasket (*Gk*) prior to chamber completion by the bottom metal retaining plate.



(Rose *et al.*: Cellophane-strip culture technique)

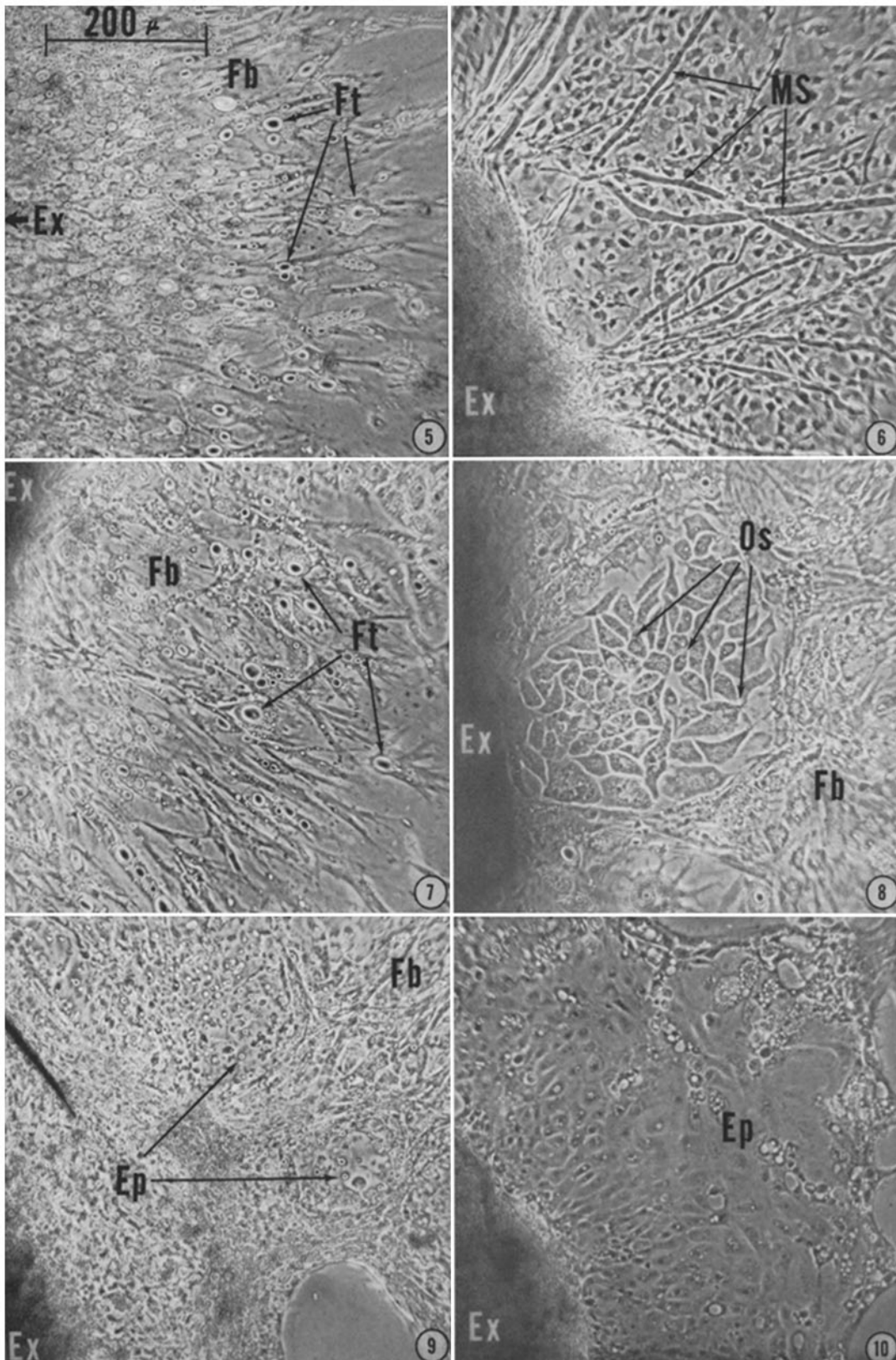
PLATE 385

FIGS. 5 to 10. These are low power phase contrast (B&L) photographs of living cells which depict the differences in outgrowth of three different tissues (muscle, bone, and thyroid) of 14-day chick embryos as they occur in multipurpose culture chambers after 10 days of cultivation. Figs. 5, 7, and 9 show outgrowths of muscle, bone, and thyroid respectively from explants which have been imbedded in clots of cockerel plasma, and Figs. 6, 8, and 10 show outgrowths of muscle, bone, and thyroid respectively from explants which have been fastened to the coverslips by the cellophane-strip technique. A magnification line on Fig. 5 may be used for all of these pictures.  $\times 125$ .

FIGS. 5 and 6. Fig. 5 shows fibroblastoid cells (*Fb*) with large fat-containing droplets (*Ft*) which is quite characteristic of all fibroblastoid outgrowths from cultures in plasma clots. Fig. 6 shows cells which are strikingly different from Fig. 5 inasmuch as they are composed of muscle straps (*MS*) emigrating from the explant. Interspersed between these muscle straps are very flat stromal cells which do not contain the prominent fat droplets.

FIGS. 7 and 8. Fig. 7 also contains cells of fibroblastoid (*Fb*) type with large fat-containing droplets. These cells cannot be differentiated from those in Fig. 5. Fig. 8 shows a nest of osteoblasts (*Os*) emigrating close to the bone fragment and into an area of fat-droplet-containing fibroblastoids (*Fb*). The osteoblasts contain alkaline phosphatase positive granules within their cytoplasm.

FIGS. 9 and 10. Fig. 9 shows a mixture of fibroblastoid cells (*Fb*) and epithelial cells (*Ep*) which have become so tightly interwoven that analysis is difficult. Fig. 10 shows a pure sheet of epithelial outgrowth (*Ep*) from an explant cultivated by the cellophane-strip technique. Note the many cytoplasmic droplets next to the nuclei which are characteristic for this type of cell.



(Rose *et al.*: Cellophane-strip culture technique)

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FIGS. 11, 12, and 13. These photographs represent living cells which differentiated as they emigrated from explants of chick 14-day embryo cultivated for 10 days with the cellophane-strip technique. Fig. 11 contains a magnification line for all three of these photographs which were taken with a 97x (B&L) oil immersion phase contrast objective.  $\times 1300$ .

FIG. 11. Here a muscle strap is coursing through the stromal cells and contains cross-striations, several nuclei (*N*), and mitochondria (*M*).

FIGS. 12 *a* and *b*. These micrographs show dense granules (*Alk*), presumably the sites of alkaline phosphatase activity. Long filamentous mitochondria (*M*) are also noted.

FIGS. 13 *a* and *b*. These micrographs demonstrate in more detail cells of the epithelial sheet in Fig. 10. Here the many numerous cytoplasmic droplets (*CD*) are evident in a juxtannuclear position. Filamentous mitochondria (*M*), cell membranes (*CM*), and phase contrast green granules (*gg*) (shown by autoradiography to be the sites of I-131 uptake) are also shown.



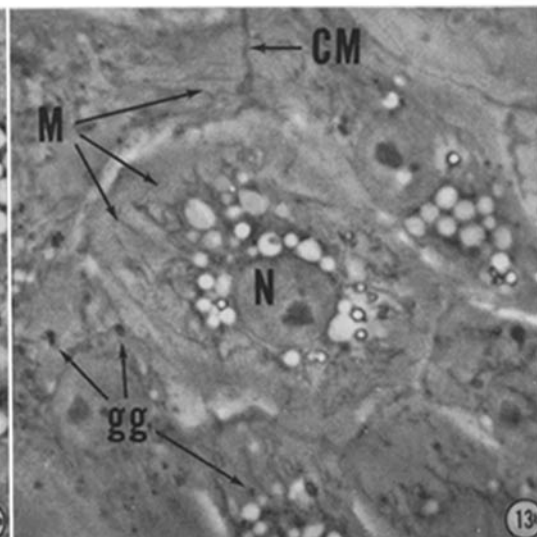
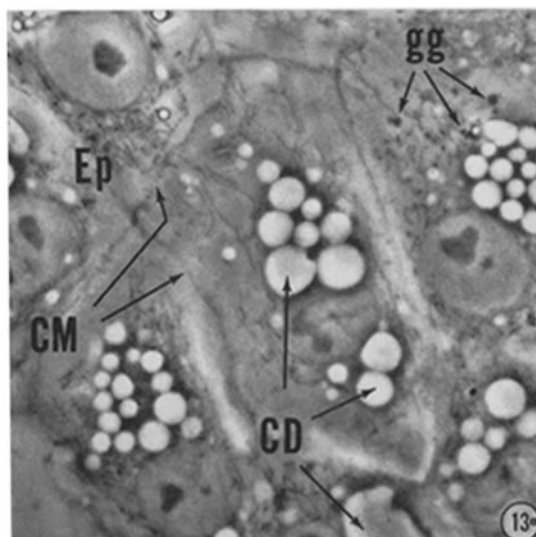
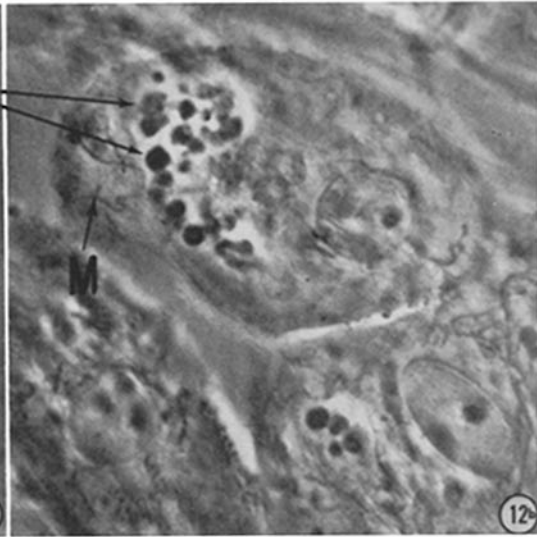
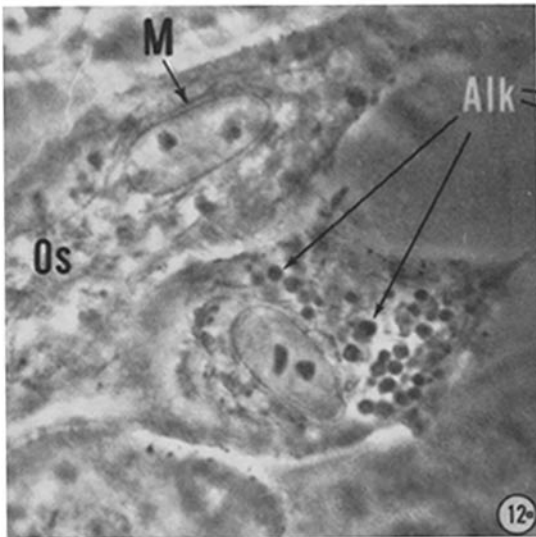
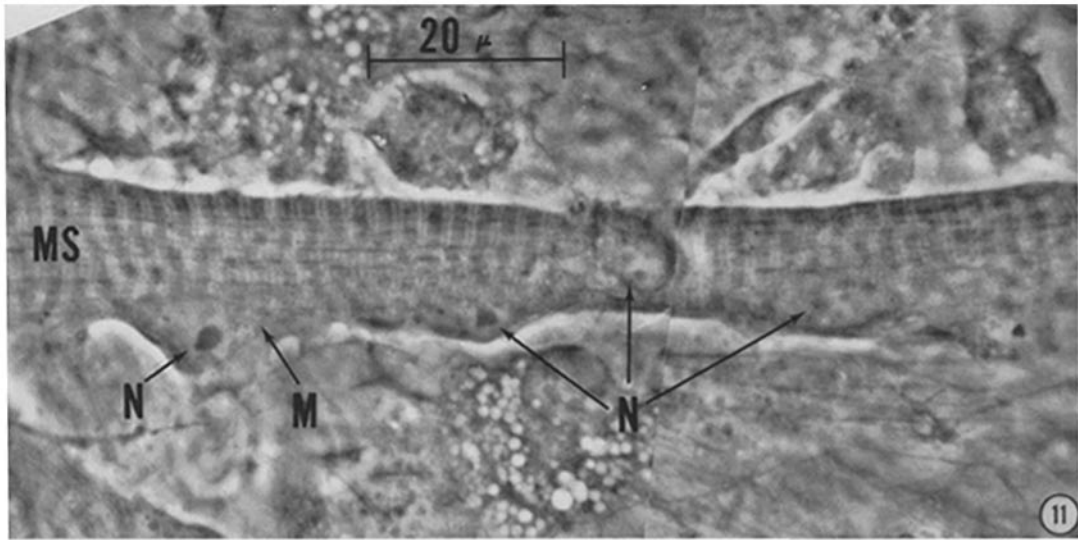
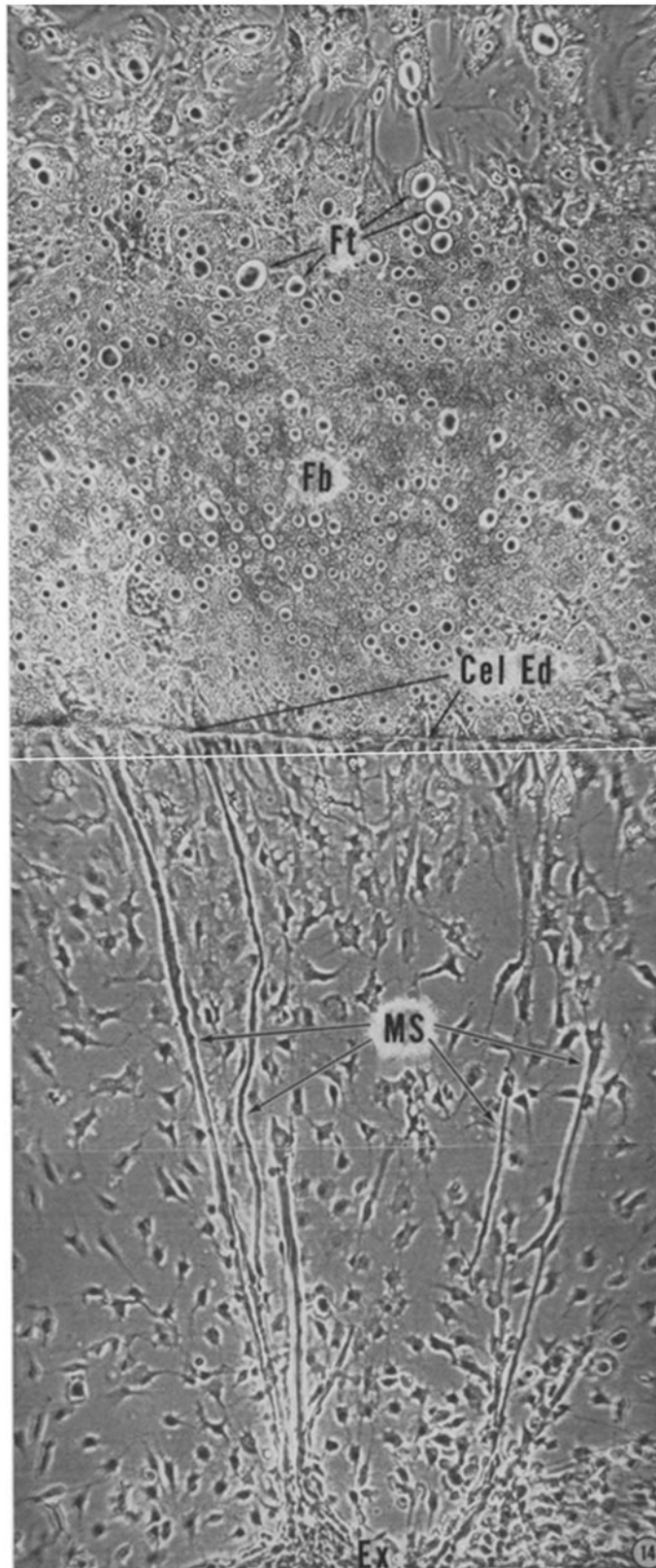


PLATE 387

FIG. 14. This montage of a muscle explant outgrowth is from a 14-day chick embryo. The outgrowth formed in a cellophane-strip culture chamber over a period of 10 days. The differentiation of muscle straps and stromal cells close to the explant and the dedifferentiation of the cell types as they emigrated from underneath the cellophane edge (*Cel Ed*) is shown. Note that the cells under the cellophane (lower half of the photograph) contained relatively few fat droplets except close to the cellophane edge (*Cel Ed*), whereas the cells which were not constrained by the cellophane are of larger and more diversified size and contained numerous huge fat droplets. Muscle straps simply did not migrate past the cellophane edge and remain in their differentiated form. There were no contractions in the cell aggregates which were not constrained by the cellophane. (B&L 10x objective, phase contrast).  $\times 125$ .



(Rose *et al.*: Cellophane-strip culture technique)