Versatile Perfusion Chamber for Living Cells and Organs

Abstract. *A microperfusion chamber, which provides controlled circulation of nutrient with chamber depths varying between 15 and 1500 microns and which permits the maintenance of transverse concentration gradients of nutrient constituents or gases, is described. The added advantage of facilitating controlled flow between adjacent chambers on a standard slide (38 × 76 mm) makes the chamber useful in the study of both plant and animal materials. Examples of such use are given.*

Continuous microscopic observation of growing and functioning cells and organs is an essential technique in biology. There are many methods for making these observations, but all have characteristic defects. Methods now in use for observing growing plant cells are particularly frustrating.

It is important to observe the cells under relatively high magnifications, which requires that they be close to the thin cover glass that separates them from the microscope objective. With animal cells, which attach themselves firmly to the surface of the culture vessel, this is not difficult. If the vessel wall is thin and cells are firmly attached in a monolayer, they can be observed with an inverted microscope, or the vessel can be temporarily inverted for observation on a standard microscope. The Carrel flask, the roller-tube, and the Rose-Sykes-Moore perfusion chambers are all designed to take advantage of this property. Plant cells, however, will not form adherent monolayers. They may settle to the bottom of the container, but ordinarily they do not attach themselves; nor, when attached, do they grow as monolayers. Instead, in unagitated liquid nutrient, they tend to grow away from the floor of the chamber in three-dimensional patterns which Gautheret has designated "pseudothalli" (*1*). This makes observation unsatisfactory even on an inverted microscope.

The chamber most widely used today for study of growing plant cells is the one devised by Marchal and Mazurier (*2*) and modified by Jones (*3*). It depends on inclusion of the culture fluid within a surrounding bath of mineral oil held under a cover glass that is supported by thin shims, usually a pair of accessory cover glasses. Cells are confined in a chamber whose depth is determined by thickness of the shims; no part of the chamber is further from the objective than 200 to 300 μ (twice the thickness of a cover glass), which permits observation with objectives of the highest magnification. Limited respiratory exchange is provided by diffusion of oxygen through the surrounding oil, and the oil also prevents evaporation of water. The culture drop is very small, never more than 0.01 ml, and it cannot be renewed.

Renewal of nutrient is important and is possible in the Rose-Sykes-Moore chambers. Attempts to confine plant cells under a viscose membrane in a Sykes-Moore chamber, as described by Rose *et al.* (*4*), so as to hold them in contact with the glass, have been unsatisfactory; the tendency to three-dimensional growth is not significantly retarded by anything as flexible as this membrane. After we tried various types of chambers (*5*) for plant materials we concluded that a new approach was necessary. Certain features of the Marchal-Jones (*3*), Buchsbaum (*6*), and Sykes-Moore (*7*) chambers have been combined in a design (*8*) which is more versatile than any of these, is simple to use, and gives excellent results (Fig. 1).

The object is to provide a means of circulating nutrient at will through an observation compartment as thin as that of the Jones chamber (*3*) and as easily handled as the Sykes-Moore chamber (*7*). The basic component is a plate of glass or plastic provided with two ports in the form of holes that are drilled into the edges at opposite sides and that communicate with the top by two openings about a centimeter apart. A cover glass is laid on this component and is supported by suitable shims. Fluid can then be fed into one port and drained off through the other, which necessitates a means of sealing

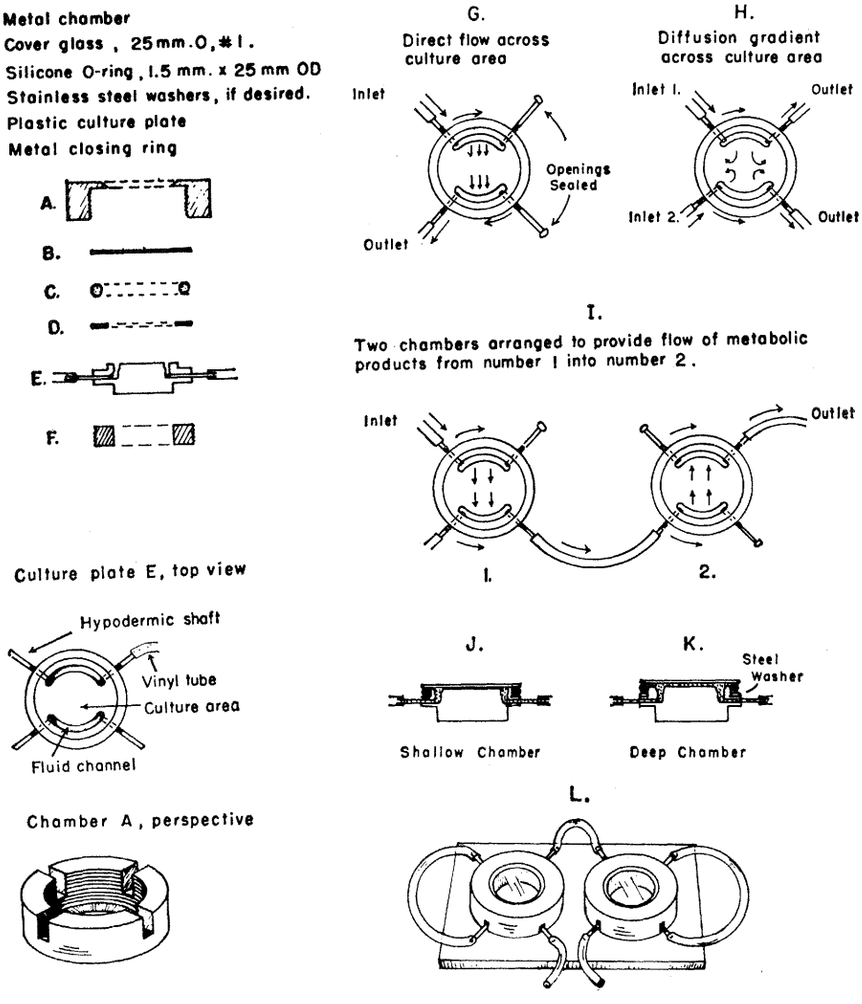

Fig. 1. Assembly of a microperfusion chamber. Details in the text.

the chamber. This was done by taking advantage of structural elements of the Sykes-Moore unit, and, with minor changes, much greater flexibility has been obtained than in any other model I have seen.

The chamber comprises five components. The culture plate consists of a disk (Fig. 1*E*) of plastic (plexiglass or better still polycarbonate) 25 mm in diameter and 6 mm thick. This is routed out at the bottom to a depth of 3 mm and a width of 3.5 mm to take the threaded sealing ring (*F*) of a Sykes-Moore chamber. The top of the disk is routed to a depth of 1 mm and a width of 1.5 mm to take a silicone O-ring (*C*). Into the edge of the 2-mm segment left between these two routed grooves are bored four 0.75-mm holes at the four quadrant positions, to a depth of 4 mm. These holes communicate with the upper surface by corresponding ports which are further connected in pairs by routed, 1-mm wide, quarter-circle grooves. The holes are threaded, and 10 mm of threaded segments of 18-gauge hypodermic needles are screwed into them. By cutting corresponding grooves into the threaded ring of a Sykes-Moore chamber (*A*), this disk, with its four lateral tubes, can be dropped into place in the ring. It is desirable to increase the depth of the chamber by 2 mm.

In setting up a culture, the metal chamber (*A*) is placed on the table, observation-side down, a 25-mm, round No. 1 cover glass (*B*) is inserted, and the O-ring (*C*) is added. The culture is placed on this cover glass. The plastic plate (*E*) is then inverted into the chamber and the closing ring (*F*) is added and screwed down; the culture is now ready for injection of nutrient.

If one wishes to circulate nutrient freely around the cells, two of the ports should be closed by capping the hypodermic tubes (*G*). Nutrient injected through one of the remaining ports will fill the quadrant groove into which the open port communicates and will then flow in a broad band across the intervening central culture area, into the opposite groove and out through the second open port. If, on the other hand, all ports are left open (*H*), different solutions can be injected into the two quadrant grooves, these solutions will flow out through their adjacent ports, and a concentration gradient will be established by diffusion across the culture area. For example, if nutrients of *p*H 5 and *p*H 9 are supplied to the two grooves and if there is free diffusion, the nutrient at the center will have a *p*H of 7 with a continuous gradient both ways.

Aeration has always been a serious problem with perfusion chambers and can be achieved by one of three methods: (i) If nutrient is fed into one channel and a gas mixture into the other, the nutrient can be continuously supplied with oxygen, at least at its margin; (ii) nutrient and gas can be fed alternately through both sides without disturbing the thin capillary nutrient reservoir over the central observation field; or (iii) the nutrient can be periodically flushed out with gas and replaced after a suitable interval.

Unlike the Sykes-Moore chamber, the depth of free space in this version is not determined by thickness of the O-ring but by the difference between this thickness and the depth of the routed upper marginal groove. With a groove depth of 1 mm and a nominal ring thickness of 1.56 mm, the difference is 0.56 mm (560 μ), and it is not difficult to tighten the ring suffi-

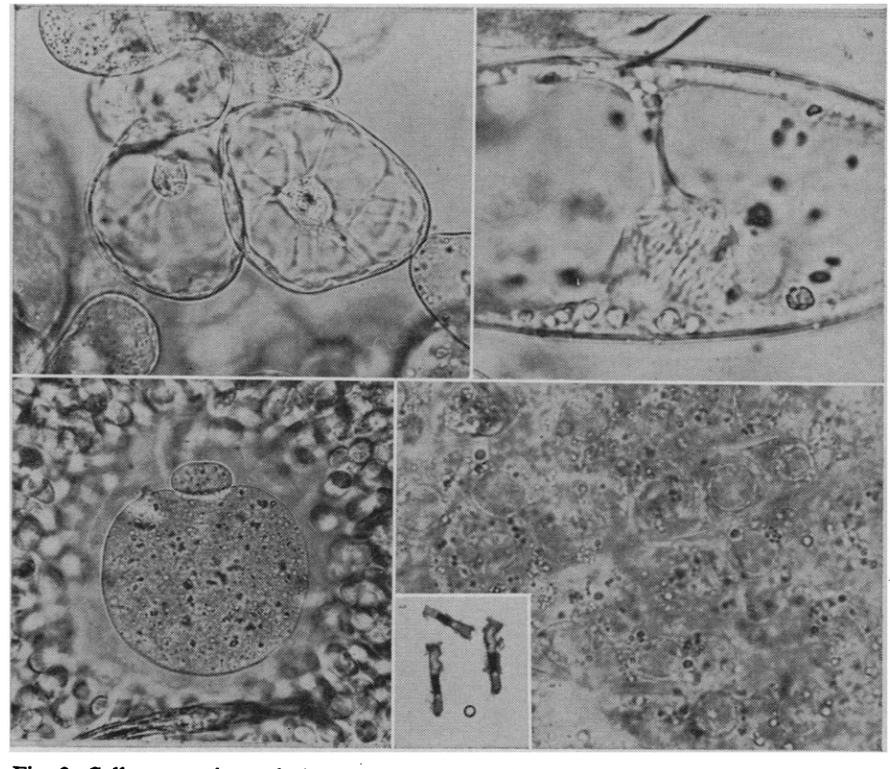

Fig. 2. Cells grown in perfusion chambers were photographed with a Wild camera and photoautomat, with the use of a built-in 7-volt lamp, with voltage stepped up to 8 volts for the moment of exposure, and Kodak Panatomic-X 35-mm film. Sensing element was set for ASA 320°; camera was set for bright-field, × 10 ocular, camera enlargement × 1.25. A long-working-distance condenser (NA, 0.52) was used for all pictures made with the compound microscope, and a Wratten No. 11 filter was employed except for the dissecting microscope picture. Because of the short camera length, true magnification on the film was one-half the "visual" magnification. All indicated magnifications were corrected for this factor. Materials shown were chosen to illustrate a variety of potential uses of these chambers. (Upper left) Cells of white spruce (*Picea glauca*). At the top and lower left are "meristematic" cells with densely granular cytoplasm and inconspicuous vacuoles. The two large cells at the center are more parenchymatous and have their nuclei suspended in a large vacuole by a system of cytoplasmic strands. Note granular texture of nuclei (× 125). (Upper right) Cell of *Picea glauca* in metaphase. This cell was followed from prophase through mitosis to completion of the daughter nuclei and of the phragmoplast (× 250). (Lower left) Mouse egg surrounded by follicular cells and showing one polar body (× 125). (Insert, lower center) Bones of foreleg of a 17-day mouse embryo, placed in a deep chamber. The picture was taken at × 3.75; a dissecting microscope (Wild) and two 7-volt lamps, one providing transmitted light, the other light reflected at a 45° angle, were used. (Lower right) Same preparation as insert, but this was photographed at × 250 and shows partially calcified cells at the contact between the central opaque portion and the adjacent more transparent subterminal portions. These two pictures show the detail that can be obtained at widely different magnifications (× 3.75 to × 250) in a single, relatively deep perfusion chamber, with the use of a standard microscope.

ciently to reduce this to 15 μ and still allow free circulation of fluid (*J*). Deeper chambers (*K*) can be obtained by inserting thin metal washers (*D*) under the O-ring; chambers of all depths between approximately 15 and 1500 μ can thus be provided.

Nutrient can be circulated through such chambers by any means desired. We first tried a "Murphy-drip," but the total volume of a 20-mm chamber, even as deep as 500 μ, is only about 0.16 ml, which means that a single "drop" of fluid would represent a complete change of nutrient, and even the slowest feasible drip would be equivalent to changing the nutrient several times an hour. Most cultures do best if the nutrient is somewhat "conditioned" by exchange of metabolic products with the tissue. This is particularly important for single-cell cultures, and it is more satisfactory to renew the nutrient for these cultures at fixed intervals, by injection with a hypodermic needle. The chamber can be closed between injections by carrying the inlet end of the tube around and attaching it to the exit tube.

Perfusion chambers have in general been handled as single units. There are, however, certain advantages in connecting them in pairs, which can be done easily with these small chambers, and in having the two chambers resting on a metal carrier (38 × 76 mm), the dimensions of a standard, wide microscope slide (*L*). Since the two chambers may have different volumes (depths), it is possible to place a large culture—up to a gram of tissue—in one chamber, to use the large culture to "condition" the nutrient, and to connect this chamber to a second shallow chamber that has been charged with single cells; or the two chambers may contain different types of cells. With animal cultures, for example, it should be possible to place pituitary tissue in one chamber and ovarian tissue in a second, to flow nutrient from one chamber to the other, and to follow directly the effects of specific metabolites. Or a plant stem tip could be placed in a deep chamber, a massive undifferentiated callus culture of the same or a different species placed in the second, and their reciprocal effects could be studied. The possible permutations are countless.

Culture plates, O-rings, and cover glasses can be sterilized by autoclaving. The chamber can be of any metal, including brass, since the nutrient does not come in contact with it. It is best to discard supply tubes after use, even though they are made of nontoxic, surgical vinyl material.

One objection to the use of these chambers in pairs, in the study of animal materials, is the tendency of animal cells to migrate in any circulated fluid. This can be obviated by placing a disk of viscose membrane over the culture and under the O-ring, and thus separate the culture from the flowing nutrient. Diffusion through such a membrane will usually be adequate for metabolic needs of the culture. If, however, there are objections to the membrane, migration can be prevented by filling the quadrant grooves with sintered nylon sponge or by introducing a Swinney-type filter into the tube connecting two chambers.

Figure 2 gives some idea of the results that can be obtained. The two upper pictures are of plant cells (*Picea glauca*) grown in shallow chambers; the lower ones are of animal materials (mouse). Equally sharp images can be obtained under oil immersion (not shown), and, if the preparations are thin and in unencumbered monolayers, phase contrast can be used. The versatility of these chambers should widely commend them for use with both plant and animal materials.

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References and Notes

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8. I wish to thank Mr. Douglas Smith of the Thorsen Machine Company, Ellsworth, Maine, for his patient cooperation in building a long series of experimental chambers before a satisfactory design was arrived at.
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1-Adamantanamine Hydrochloride: Inhibition of Rous and Esh Sarcoma Viruses in Cell Culture

Abstract. *1-Adamantanamine hydrochloride, added to chick embryo cells, inhibited focus production upon subsequent inoculation of the cells with Rous and Esh sarcoma viruses. Addition of the chemical to suspensions of Rous and Esh viruses before they were added to chick cell cultures did not inactivate the viruses; and the addition to chick cells did not prevent plaque formation following their inoculation with Newcastle disease virus. This indicates that cells treated with 1-adamantanamine hydrochloride are capable of supporting viral replication, and these observations suggest that the drug inhibits growth of two oncogenic viruses by prevention of virus penetration, but not by a virucidal effect or cell intoxication.*

1-Adamantanamine hydrochloride (amantadine HCl) inhibits certain myxoviruses; that is, several strains of human influenza and parainfluenza viruses in addition to their animal moieties. Other viruses, rubella and pseudorabies, are also sensitive in one or more systems (*1, 2, 3*).

Avian oncogenic viruses share many characteristics with myxoviruses: they are ether labile, sensitive to *p*H 3.0, within the size limits of 80 to 200 mμ, and contain pentose nucleic acid (*4*). Hence, it appeared pertinent to investigate the action of amantadine on these viruses in a quantitative cell system.

In the assay, patterned after that of Rubin (*5*), secondary chick embryo fibroblasts were used for focus production by Rous sarcoma virus (RSV) (Bryan strain) and Esh sarcoma virus (ESV), a recent field isolate (*6*). Growth medium (*5*) was modified to include Eagle's basal medium as well as medium 199; calf serum was substituted for chicken serum.

Immediately after adding amantadine HCl, tubes containing the chick cell suspension were placed in a water bath (37°C) for 15 minutes. After incubation, the drug-cell suspension was pipetted into small plastic petri dishes and virus was added to all plates within 5 minutes. Next day, cultures were overlayed with agar that contained amantadine. After incubation for 7 days in a CO_2 incubator (5 to 7 percent CO_2 and 90 to 98 percent relative humidity) virus-induced foci were counted to quantitate inhibitory activity.