# Cell Culture on Artificial Capillaries: An Approach to Tissue Growth in vitro

Abstract. *Artificial capillaries perfused with culture medium provide a matrix in which cells can attain tissue-like densities in vitro. Products secreted into the medium can be measured as indicators of cell function or may be recovered for other purposes without disturbing the culture.*

Certain characteristics of many cell lines change as a function of in vitro culture density (*1*). The in vivo behavior of normal or neoplastic cells, therefore, may be impossible to evaluate in vitro unless cell densities comparable to the in vivo state are obtained. Attempts to produce tissues in vitro to satisfy this requirement have met with limited success (*2*). Diffusion of nutrients and cell products within tissue appears to be a major obstacle to attaining the desired densities; the effective diffusion lengths are less than 300 $\mu$m for small molecules (*3*) and are likely to be more limited for proteins (*4*). Periodic or continuous replacement of nutrient medium to enhance diffusion causes sudden alteration of the fluid composition immediately adjacent to the cells (*5*) or prevents accumulation of essential cell products (*6*). Such variations of the cellular microenvironment may alter cellular controls needed for the formation of tissue masses. We report here the development of a new method that solves these problems, at least in part, and permits the growth in vitro of cells to tissue-like densities within an artificial capillary bed.

The capillary bed was simulated by cell culture units within a perfusion circuit (Fig. 1). Two types of units were used. The first was a commercially available ultrafiltration cell (c/HFU-1/20, Dow Chemical) composed of 100 cellulose acetate capillaries (9 cm long) sealed in a standard 8-mm glass tee, which provided 46 cm$^2$ of membrane area in a space of 1.5 cm$^3$. Each capillary had a 250-$\mu$m outer diameter and a 25-$\mu$m wall thickness that nominally limited diffusion to substances having molecular weights less than 30,000. The second type of culture unit was composed of 30 polymeric capillaries (XM-50, Amicon) mixed with 30 silicone polycarbonate capillaries (Dow Chemical). The capillary bundle was pulled into a 90- by 8-mm glass shell (Fig. 1a) having two glass side ports (Fig. 1f). Both bundle ends were then closed with a suture tie and immersed in liquid silicone rubber (RTV-11, General Electric) diluted 3:2 with 360 Medical Fluid (Dow Corning) and catalyzed with stannous octoate (Nuocure 28, Tenneco). The capillary bundle was trimmed flush to the shell ends, and tapered glass fittings were attached to accept the silicone rubber tubing. The XM-50 capillary had a 340-$\mu$m outer diameter with a 75-$\mu$m wall thickness that nominally limited diffusion to substances having molecular weights less than 50,000; the silicone polycarbonate capillary, with approximately 260-$\mu$m outer diameter and 40-$\mu$m wall thickness, was permeable to gases and was therefore included to increase oxygen and carbon dioxide transport into each culture unit.

The perfusion circuit was sterilized in ethylene oxide for 6 hours and exposed to room air for 1 to 2 days before use. The equipment was then placed in a 37°C incubator at approximately 90 percent humidity, and both the perfusion circuit and the shell surrounding the capillary bundle were flushed with culture medium for 1 to 2 days to remove remaining traces of ethylene oxide. The perfusion medium reservoir was emptied and refilled with fresh medium, and cells, suspended in 1 to 2 ml of the same type of medium, were injected through a shell port onto the perfused capillary bundle. The cells were allowed to settle and adhere to the capillaries for 1 day, after which the extracapillary medium within the shell was replaced with fresh medium to remove unattached cells and cell debris.

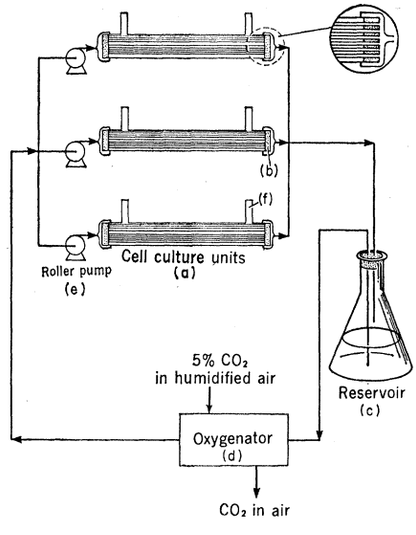


Fig. 1. Perfusion circuit. A cell culture unit (a) consisted of a bundle of one or more types of hollow fibers (capillaries) sealed into each end of an 8-mm glass shell by silicone rubber or epoxy resin (b). Units may be arranged in parallel as shown or in series. Nutrient medium stored in a 125-ml reservoir flask (c) was oxygenated and brought to the appropriate *p*H by exposure to a humidified mixture of 5 percent $CO_2$ and air in a Dow Corning Mini-lung (d) (*13*) before being pumped through each capillary bundle (pump RL-175, Holter) (e). Components were connected by silicone rubber tubing (3.2 mm outer diameter). Cells were inoculated onto the capillary bundles through shell side ports (f).

When some experiments were terminated, the extracapillary medium was replaced by a warm 4 percent agarose suspension in normal saline through a shell port to hold the cells in place. After cooling, the glass ends of the shell were fractured, the exposed ends of the bundle were cut, and the agarose-encased capillary bundle was removed and placed in cold 10 percent formalin. Sections perpendicular to the long axis of the bundle were made 2 days later and stained for histologic study by standard techniques

Mouse fibroblasts (L-929) were cultured on hollow cellulose acetate capillaries. A suspension of 200,000 cells was inoculated onto each capillary bundle perfused at 0.7 ml/min by medium (*7*) that was replaced every 1 to 4 days; the extracapillary medium (*7*) was replaced weekly. Small nodules were grossly visible on the capillaries about 2 weeks after inoculation; their cell count was estimated from DNA content to be $17 \times 10^6$ (*8*). The nodules continued to enlarge, many attaining diameters of approximately 1 mm after 28 days of incubation (Fig. 2a). Histologic sections showed necrosis in the central portions of most nodules (Fig. 2b).

Human choriocarcinoma cells (JEG-7) (*9*) were cultured on mixed bundles of XM-50 and silicone polycarbonate capillaries. A suspension containing approximately $1.5 \times 10^6$ cells was injected into each of three parallel culture units. The extracapillary and perfusion media (*10*) were replaced simultaneously every 1 to 2 days. Periodic analyses (*11*) of the perfusion medium for human chorionic gonadotrophin (hCG) showed an initial lag in hCG production, after which the rate of production doubled every 1.6 days (Fig. 3b). Subsequent cultures were perfused with

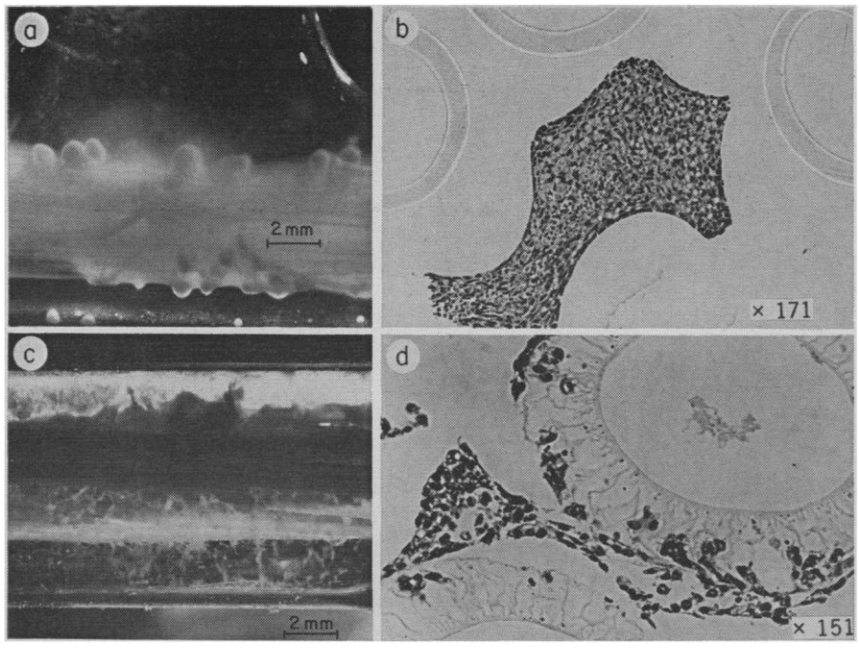


Fig. 2. (a) Nodules of L-929 cells on cellulose acetate capillaries after 28 days of culture. (b) Histologic section through capillary bundle shown in (a). Phase contrast was used to show the unstained, transparent capillary; the nuclei appear very dark when this technique is used. Edges of the cell mass are scalloped due to growth between capillaries. Spaces between the cell mass and capillaries are artifacts of fixation. Necrosis is seen in areas most distant from capillaries. (c) JEG-7 cell growth on a mixed bundle of 30 XM-50 and 30 silicone polycarbonate capillaries after 28 days of culture. (d) Histologic section (phase contrast) through the capillary bundle shown in (c). Multilayers of cells have invaded the porous outer surface of the XM-50 polymeric capillary and bridged to the adjacent polymeric capillary.

medium (*12*) at 5 ml/min without replacement of extracapillary medium. These cultures produced hCG at a rate that doubled every 1.2 days (Fig. 3a), in accord with the cell doubling times (1.1 to 1.3 days) observed in standard monolayer cultures in our laboratory. The hCG production rate after 28 days of culture was equivalent to that of $217 \times 10^6$ cells in monolayer culture (*9*), but required less than 3 $cm^3$ of actual culture space (Fig. 2c). Thus, the cell density obtained in this experiment clearly approaches that of tissue in vivo if the rates of hCG production are the same in capillary and monolayer culture. An alternative explanation is that the efficiency of hCG production may be increased by growth within the capillary bundle. Further work is in progress to resolve this question.

Histologic sections through the bundle after 28 days of culture showed multilayer cell growth with invasion of the porous capillary surfaces and bridging between adjacent capillaries (Fig. 2d).

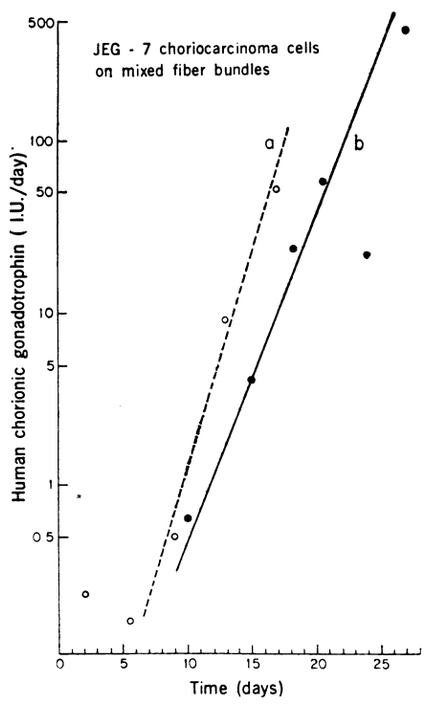


Fig. 3. Rate of hCG production as a function of duration of JEG-7 cell culture on mixed capillary bundles (*I.U.*, international units). Open circles (line a) are data for cultures perfused with 5 ml of medium (*12*) per minute without replacement of extracapillary medium. Solid circles (line b) denote cultures perfused with 0.7 ml of medium (*10*) per minute with periodic replacement of extracapillary medium.

Several conclusions can be drawn from these studies. Measurements of hCG that had diffused into the perfusion medium showed that concentration changes of nutrients or secretory products can be monitored as an indication of cellular function, and that valuable products such as hormones can be retrieved from the perfusion medium without disturbing the culture. The high ratio of surface area to volume in this system should allow large numbers of cells to be cultured in a relatively simple apparatus with considerably less space and equipment than are required for existing commercial culture techniques. Formation of the cell multilayers and nodules as described indicates that the artificial capillary system affects the culture environment in ways that allow cells to grow to solid tissue densities in vitro.

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**References and Notes**

1. E. Miedema and P. F. Kruse, Jr., *Biochem. Biophys. Res. Commun.* **20**, 528 (1965); P. F. Kruse, Jr., L. N. Keen, W. L. Whittle, *In Vitro* **6**, 75 (19→ H. Rubin, *J. Cell Biol.* **51**, 686 (1971); A. A. Tuffery, *J. Cell Sci.* **10**, 123 (1972); C. E. Cass, *J. Cell. Physiol.* **79**, 139 (1972); J. B. Warshaw and M. D. Rosenthal, *J. Cell Biol.* **52**, 282 (1972); J. B. Griffiths, *J. Cell Sci.* **10**, 515 (1972).
2. J. Leighton, *J. Nat. Cancer Inst.* **12**, 545 (1951); P. F. Kruse, Jr., and E. Miedema, *J. Cell Biol.* **27**, 273 (1965); J. Leighton, G. Justh, M. Esper, R. Kronenthal, *Science* **155**, 1259 (1967).
3. R. H. Thomlinson and L. M. Gray, *Brit. J. Cancer* **16**, 841 (1955); I. F. Tannock, *ibid.* **22**, 258 (1968).
→ R. Holmes, *J. Cell Biol.* **32**, 297 (19(→ T. Puck, C. A. Waldren, C. Jones, *Proc. Nat. Acad. Sci. U.S.A.* **59**, 192 (1968); J. Folkman, *N. Engl. J. Med.* **285**, 1182 (1971).
→ C. Ceccarini and H. Eagle, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 229 (1971).
6. H. Eagle and L. Levintow, in *Cells and Tissue in Culture*, E. N. Willmer, Ed. (Academic Press, London, 1965), vol. 1, pp. 277–296.
7. The medium consisted of 90 ml of Eagle's spinner modified basal essential medium, 10 ml of fetal calf serum, 5000 units of aqueous penicillin, and 5 mg of streptomycin.
8. K. Burton, *Biochem. J.* **62**, 315 (1956).
9. P. O. Kohler, W. E. Bridson, J. M. Hammond, B. Weintraub, M. A. Kirschner, D. H. Van Thiel, *Acta Endocrinol. Copenhagen*, Suppl. 153 (1971), p. 137.
10. The medium consisted of 83.3 ml of Ham's F-10 medium, 13.5 ml of horse serum, 3.2 ml of fetal calf serum, 5000 units of aqueous

penicillin, 5 mg of streptomycin, 0.5 mg of insulin, and 0.62 mg of cortisone acetate.
11. W. D. Odell, P. L. Rayford, G. T. Ross, *J. Lab. Clin. Med.* **70**, 973 (1967).
12. This medium contained 83.3 ml of Ham's F-10 medium, 13.5 ml of horse serum, 3.2 ml of fetal calf serum, 5000 units of aqueous penicillin, and 5 mg of streptomycin.
13. P. M. Gullino, in *Organ Perfusion and Preservation*, J. C. Norman, Ed. (Appleton-Century-Crofts, New York, 1968), pp. 877–898.
14. We thank the Dow Chemical Co. for the silicone polycarbonate capillaries, R. Cross of the Amicon Corp. for the XM-50 capillaries and technical advice, and W. R. Kidwell of the National Cancer Institute for his helpful suggestions.

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## Chemical Trail-Following by Flying Insects: A Mechanism for Orientation to a Distant Odor Source

Abstract. *An aerial trail of odorous pheromone molecules extends downwind from a female pink bollworm moth that is receptive for mating. Males apparently sense the boundaries of the trail during their characteristic zigzag flights across it. Contrary to previous beliefs, the mechanism by which the males steer toward the odor source does not require a sensing of wind direction.*

Insects of many species identify their mates, food, and egg-laying sites by olfactory chemical cues. The molecules diffuse or drift on air currents away from their source, often providing information to the insect on the direction of the source.

Within a few centimeters of an odor source, an insect may be able to detect a steep concentration gradient of molecules and orient its body axis accordingly. However, because of the turbulence that is characteristic of moving air, a chemical gradient probably cannot provide useful directional information over more than a few centimeters (*1*). An insect detecting molecules many meters downwind must use another mechanism to steer toward the odor source.

Anemotaxis (orientation to an air current) has been shown to play a key role in the steering of certain insects to a distant odor source (*2*). The evaporating odor molecules form an elongated, often irregularly shaped cloud or plume moving in a downwind direction. An insect that orients anemotactically turns its body axis into the wind when it is stimulated by the odor. As it proceeds in this upwind direction, the odorous air stream might be lost; crosswind casts may then occur, presumably maximizing the likelihood of the insect again encountering the odorous air and continuing its upwind flight. In recent years, anemotaxis has been accepted by many investigators as the only available mechanism for the orientation of a flying insect to a distant odor source (*3*).

We have examined the method used by a small moth species, the pink bollworm, *Pectinophora gossypiella,* in steering toward an odor source and have found that sensing of the direction of air movement is not necessary. An alternative mechanism for orientation to a distant odor source is proposed.

A Plexiglas flight tunnel was constructed, 183 cm long by 61 cm wide by 61 cm high, with screens (six meshes per centimeter) covering both ends (Fig. 1). Air flow was obtained by placing one end of the tunnel adjacent to an exhaust hood. Prior to introduction of the odor source, a cage containing ten male moths was attached to the center of the screen at the downwind end. The males were conditioned for at least 10 minutes to an air velocity of 7 cm/sec and a light intensity of 4 lux. The odor source was sex pheromone, prepared by extracting female abdomen tips in ether. Pheromone extract equivalent to 0.1 female was pipetted into one end of a copper tube 2 mm in inside diameter. The other end was connected to a flexible air line delivering 0.25 $cm^3$ of air per second. During a test, the tube was positioned so that evaporating pheromone molecules were released into the air in the center of the upwind end of the tunnel. When odor molecules activated the males at the downwind end, a trapdoor that covered the front of their cage was opened.

The males were considered to be orienting within the pheromone plume if they flew in an upwind direction through a square wire hoop (30 $cm^2$) suspended in the center of the tunnel 30 cm downwind from the pheromone source. The hoop occupied 25 percent of the cross-sectional area of the tunnel. Prior calibration of the system with visible smoke in air moving at 7 cm/sec (*4*) indicated that a typical plume was approximately 14 cm in diameter and 25 cm in diameter when it passed through the hoop and the downwind screen, respectively.

Three experimental conditions were evaluated: condition 1, a pheromone plume in moving air; condition 2, a pheromone plume in still air; and condition 3, no pheromone plume in still air. Condition 1 was obtained by releasing pheromone into air moving through the tunnel at a velocity of 7 cm/sec. In condition 2 a pheromone plume was formed in air moving at 7 cm/sec, but the air flow was stopped as soon as a moth entered the plume on the downwind end. At that time, solid baffles were moved into position on both ends of the tunnel and the release of pheromone from the source was terminated. Prior experience with visible smoke showed that movement of the air in a downwind direction ceased immediately and the plume remained essentially intact and suspended in space for about 20 seconds before it started to break up. Condition 3 was obtained by releasing only a 5-second pulse of pheromone from the source into air moving at 7 cm/sec.

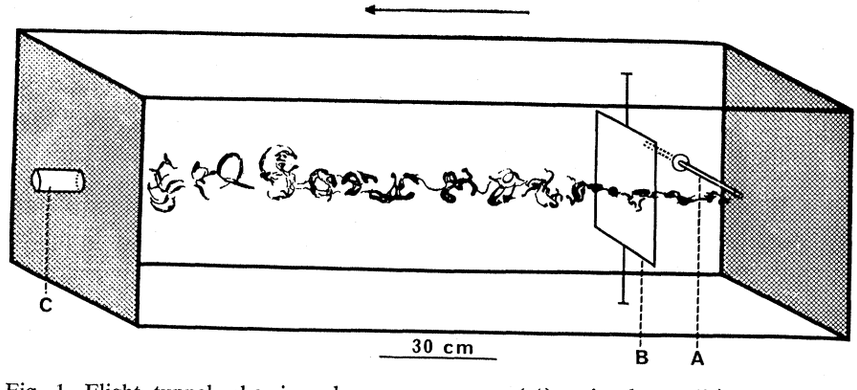


Fig. 1. Flight tunnel, showing pheromone source (*A*), wire hoop (*B*), and release cage for male moths (*C*). An artist's representation of an odor plume, based on an actual photograph of a visible smoke plume, is drawn in the tunnel. Screens on the ends of the tunnel are represented by cross hatching. The arrow indicates the direction of the wind.