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Exploring Processes of Organization of Normal and Neoplastic Epithelial Tissues in Gradient Culture

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Abstract The biology of animal cells in culture is often studied in individual cells or in sheets of cells. The relevance of such studies to the intact animal is unclear, since the spatial conditions encountered by cells in animals is one of dense three-dimensional masses of cells, with limits to migration, and with gradients both of diffusion of metabolites and of morphologic maturation. These spatial requisites have gradually been met in culture. A brief account describes sponge matrix culture for three-dimensional growth and unilaminar, bilaminar, and radial histophysiologic gradient cultures. Some of the common neoplastic abnormalities of surface epithelial tissues are considered. Proposals for investigating the histokinetic mechanisms regulating some epithelial tissue processes are suggested. In the most recent development of gradient culture methods, a thin permeable transparent collagen membrane is intrinsically strengthened by producing a waffle membrane pattern for histophysiologic gradient culture. © 1994 Wiley-Liss, Inc.

Key words: chicken plasma clot, histophysiologic gradient culture, neoplastic blockade, collagen waffle membrane, epithelial tissues

INTRODUCTION

The three-dimensional structure of normal tissue in nature must be of critical importance for durable function since it is precisely maintained. The structure of carcinomas, the basis of their classification, usually recalls the tissue of origin. The structure of these tumors is also maintained, though not with the rigor of their antecedent normal tissue. The propagation of cancer must be an almost equally well-regulated process since increasing numbers of distinctive, similar groups of cells arise [Leighton, 1959; Leighton et al., 1960]. Secondary growths appear as recognizable variations of tissue patterns found in the original tumor.

I have been interested in the structural-functional organization of epithelial tissue, an inextinguishable interest ignited by Jean Oliver, my Professor of Pathology when I was a medical student in the 1940s. A part of my research effort has focussed on defining the essential conditions found in nature that must be provided for cells in culture, whether of normal or neoplastic origin, if they are to reconstitute the tissues they formed in nature.

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In this essay I have three goals. One is to describe some of the spatial configurations that epithelia regularly attain in normal tissue and in the course of neoplastic development. Another is to consider several conceptual spatial imperatives that I think are essential to the valid reconstitution in culture of the spatial conditions seen in nature, and methods for complying with these requisites. Finally I pose some questions for study that emerge from consideration of epithelial tissue organization and suggest a few lines of investigation of these issues with histophysiologic gradient culture methods.

CONFIGURATION OF EPITHELIAL TISSUE BOTH NORMAL AND NEOPLASTIC

Stratified epithelium, e.g., the epidermis, the lining of the esophagus, and the lining of the urinary bladder, are most easily depicted in a drawing. Other more anatomically complex epithelial organs are not considered here.

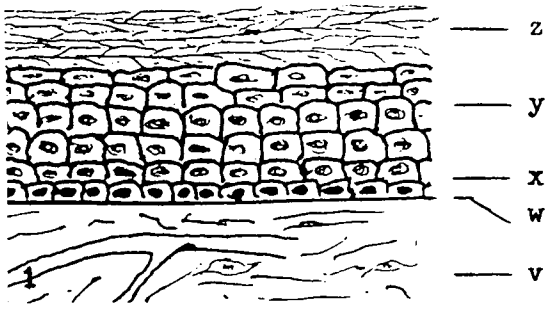
In Figure 1, a process of progressive cell maturation is depicted as the cells from the basal surface near the stroma ascend to the mature free surface of the epithelium. The epithelium is polarized; a gradient of maturation is observed accompanied by a gradient of diffusion of metabolites from the most basal to the most superficial cells. Constituents essential for the nutrition of

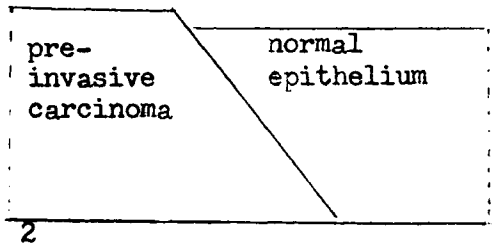
the layers of cells diffuse from the basal surface to the free surface, and concurrently metabolic products of the stratified cells diffuse through the basal cells to the stroma, to be removed by the vascular bed. The polarity of the metabolic gradient is one durable element in the physiologic process, whether the circulation of blood is normal or deficient.

In considering the steps in the eventual appearance of neoplasia, the skin and the cervix of the uterus are convenient sites since their superficial aspects are readily available for clinical scrutiny. As changes toward neoplasia develop, a chronology can be documented. The terms used to denote the sequence are hyperplasia, dysplasia, and preinvasive cancer, or carcinoma-in-situ. In this last category, the population of cells is clearly abnormal, and the sequence of morphologic cellular maturation from the basal to the free surface is all but gone.

Another quality of the epithelial cells that line the body's outer and inner surfaces is their continuity. The cells of one epithelial type share interepithelial boundaries with other cells on the opposite side of the boundary. The lip of the mouth is one example. Two others are the junction of the esophagus and the stomach, and the endocervix and the vaginal cervix, common sites of neoplastic change.

When carcinoma-in-situ is found in an area of the transformation zone in the uterine cervix, the border between the neoplastic and non-neoplastic cells is a new interepithelial boundary. In many instances, on microscopic section of this boundary a plane of separation appears at an angle between the plane of the interface of stroma and epithelium, suggesting that the neoplastic epithelium replaces normal epithelium by progressive undermining, separating the normal epithelium from its supporting stroma, a neoplastic blockade (Fig. 2) [Leighton, 1968]. Occasionally Pagetoid invasion is seen in the epidermis of the nipple in breast cancer, where scattered single or groups of large cancer cells infiltrate the epidermis.





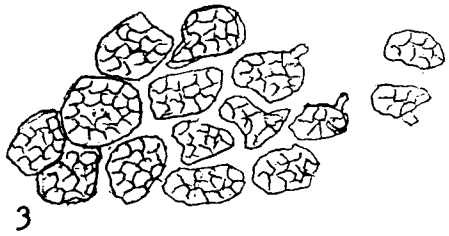


Fig. 1. Diagram depicting a stratified epithelium such as the skin. The dermis, v, is composed of dense connective tissue with blood vessels, collagen bundles, extracellular material, and connective tissue cells. Interface between the dermal stroma and the basal layer, w, is distinct, and thin. Basal zone of the epidermis, x, is the site where the initiation of renewal takes place. An intermediate zone of cell maturation is labeled y. Zone z is superficial and consists of flattened cells in a shingle-like arrangement, protecting the deeper layers.

Fig. 2. Drawing depicting interepithelial boundary between preinvasive cancer (carcinoma-in-situ) and normal stratified epithelium. In many such boundaries, the carcinoma appears to undermine the normal epithelium, separating the normal epithelium from the parenchymal/stromal interface.

Fig. 3. Diagram depicting a carcinoma as round groups of tumor cells invading a normal tissue. Aggregates of cancer cells increase in number as the process progresses. Note that the aggregates have a family resemblance.

When considering cancer invading the stroma, an essential local finding is that of increasing numbers of groups of cancer cells through time. The carcinoma cells may appear in large groups, branching cords, globular aggregates of cells, or as masses of single cancer cells infiltrating the stroma (Fig. 3).

Study of the morphology of stratified epithelium in vitro requires conditions where histophysiologic gradients prevail. When rats receive a subcutaneous injection of air and a mince of normal rat bladder, in less than 2 weeks the cavity is filled with fluid and is lined by orderly stratified urothelium, producing a heterotopic urinary bladder [Roberts et al., 1974]. No viable epithelium occurs in any other configuration.

My major experimental experience has been with neoplasia of stratified epithelia, with two exceptions, the dog kidney cell line MDCK [Abaza et al., 1974; Leighton et al., 1969, 1970, 1972; Takeuchi et al., 1974], and late stage carcinoma of the human ovary. MDCK, derived from normal dog kidney, has been appreciated for many years as a neoplastic epithelium. In three-dimensional matrix culture it grows as a papillary adenocarcinoma, its tissue pattern resembling some carcinomas of the kidney. MDCK is polarized in one of two patterns in culture. In the substance of a chick plasma clot it forms glands with microvilli lining the lumen. Suspended in liquid medium it grows as cysts with microvilli on its outer surface. On cine time lapse study suspended cysts are seen to be engaged in active transport.

The changes that occur on histologic examination of glandular tissue during the development of neoplasia are, in a general way, similar to those seen in stratified epithelium. There is multilayering of cells, abnormalities of polarization, the appearance of bizarre glands lined by large cells, and the extension of groups of neoplastic glands into the stromal substance of the organ deep to the lining layers of the usual epithelium. As in epidermoid carcinoma, when the tumor invades the stroma abnormal groups of neoplastic cells increase in number. Should the tumor be eventually disseminated, the remote colonies appear as many neoplastic aggregates replacing normal tissue. The manner in which the number of aggregates of cancer cells increases is unknown [Leighton, 1969].

The spatial imperatives for rational tissue culture procedures require providing in culture some essential conditions always present in nature. A quote from antiquity is appropriate: "Recognize what is before you, and what is hidden will be revealed unto you." In my view the essentials for rational tissue culture that have been recognized up to the present are 1) three-dimensional growth of dense populations of cells; 2) restraints and barriers to the free migration of cells; 3) settings for the establishment and recognition of gradients of diffusion and maturation, e.g., histophysiologic gradients; and 4) conditions in which there is continuity of epithelial membranes.

The first matrix for tissue culture, in general use from 1910 to 1950, was a chicken fibrin clot. Subsequently, the use of other gels became fashionable such as agar and other marine colloids, collagen gel, umbilical cord, and currently ECM (extra cellular matrix) or Matrigel (Becton Dickinson Labware, Bedford, MA). As a student of culture techniques during the plasma clot generation, and in recognition of the merit of the work of many others, my own work started out using plasma clots. Nature employs a fibrin clot in the tissue culture in vivo called wound healing.

One of the difficulties in studying organotypic growth in transparent fibrin clots in culture is the disruption of tissue architecture that takes place as enzymes produced by the tissue liquefy the plasma clot. The sponge matrix technique was developed so that fragments of tissue could be cultivated on a double matrix, one of fibrin clot reinforced by cellulose sponge. When clot lysis occurred, the degree of disruption of organized new tissue was reduced, since the new growth was anchored to the insoluble cellulose sponge in many places. Photomicrographs illustrated this point well in earlier publications [Leighton, 1951, 1954]. The introduction of soluble collagen facilitated the use of a complex matrix in the form of a collagen coated cellulose sponge [Leighton et al., 1967, 1968; Leighton, 1973]. When the trabeculae of cellulose sponge are coated completely with a thin layer of liquid collagen, which is then dried and made insoluble, the surfaces subsequently presented to the cells for their attachment is one of collagen, not of cellulose.

Two things were observed repeatedly that contributed to the subsequent development of gradient models. Various tissues of the chick embryo were cultured on pieces of collagen coated cellulose sponge, measuring 1.5 × 4 × 6 mm, cemented with plasma clot to the glass surface of

the culture tube. The dense growth of the most histotypic aspects of the tissue was invariably at the medium bathed surface of the piece of sponge. Another harbinger of developments ahead took place as epithelial tissues of the chick embryo were cultured where the technique of initial preparation had been imperfect, when there were obvious air bubbles trapped in the fibrin clot within the sponge interstices. After a few days in culture the air in these bubbles was replaced by fluid. After a week or two in culture, histologic sections showed these cysts to be lined by embryonic epithelium, the cystic chambers producing polarized patterns in epithelium, reminiscent of the heterotopic bladders induced in the subcutaneous tissue of rats, as cited earlier.

In these circumstances, where transplanted or cultured stratified epithelium produced polarized stratified epithelium in new sites, the incentive to provide standard conditions for histophysiologic gradient culture was increased. Unilaminar histophysiologic gradient culture became the first model that worked well.

METHODS FOR COMPLYING IN CULTURE WITH THE SPATIAL IMPERATIVES OF NATURE

When a collagen coated cellulose sponge is used in combination with plasma clot, the growth in the presence of clot liquefaction maintains a better anchorage to the collagen membrane covering the cellulose trabeculae than to uncoated cellulose structures. When collagen coated sponge is used without a fibrin clot, proliferating migrating cells readily enter the interstices, producing a dense tissue in the most aerobic part of the sponge, on the surface zone closest to the atmosphere in the chamber. These procedures have been described previously in detail [Leighton, 1973].

Unilaminar histophysiologic gradient cultures are prepared by culturing fragments of tissue on a thin permeable transparent membrane of collagen with appropriate reinforcement to keep the membrane flat and arranged so that all of the exchange of metabolites for the cultured tissue is by diffusion across the collagen membrane. This setting was attained at first using a disc of cellulose sponge with a large central perforation, to which a collagen membrane was cemented. As the culture is prepared, an explant of tissue is cemented to the floor of the chamber with plasma clot. The sponge is then inverted and placed in a plastic capsule with a single aperture at its top. The membrane, now the roof of the culture chamber, is in contact with the rim of the plastic aperture. All metabolic exchange takes place at the aperture, across the thin collagen membrane (Fig. 4). Most of our studies with this unilaminar procedure have concerned normal and cancerous urothelium.

Bilaminar histophysiologic gradient cultures were prepared by using two discs of cellulose sponge, each with a central perforation, and closing one side of each opening with a collagen membrane. In preparing cultures, the tissue is sandwiched between two contacting collagen membranes, and the sandwich is placed in a plastic capsule with an aperture on both the top and bottom face of the chamber (Fig. 4). Rat urinary bladder cultured in this system for 10 to 15 days gave rise to a flattened single cystic growth lined with polarized stratified urothelium, a microbladder [Leighton et al., 1984b].

The conception of a radial gradient culture chamber was inspired by the microbladder. Initially we used a collagen cylinder prepared commercially [Leighton et al., 1985]. The requirements were a thin walled, permeable, relatively transparent tube, measuring about 2 mm in internal diameter. Pieces of collagen tubing, about 2.5 cm long, were ligated at one end. The inoculum was placed in the open end, moved toward the ligature, and then the open end was ligated. The sausage shaped structure was placed in a glass chamber with medium and incubated on a rocker. Subsequently, we prepared collagen tubes in our laboratory, reinforcing the fragile thin collagen membrane by incorporating a nylon mesh in the membrane. The methods of preparation have been completely described [Leighton et al., 1984]. Now the last spatial imperative was fulfilled. As an inoculum of immortal epithelial cells proliferated, stratified, and migrated, the sausage shaped culture chamber was completely lined with epithelium. There were no free edges.

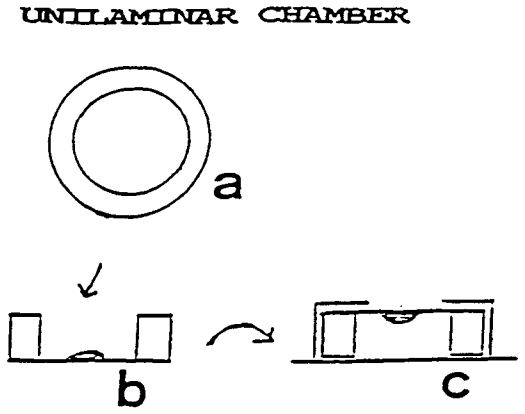
The nylon mesh does create small artifacts when cutting sections of the culture chambers embedded in paraffin. However, a collagen cylindrical membrane without reinforcement was too fragile to handle easily. Culinary experience suggested a solution. When pancake batter is used in the preparation of flat cakes, artifacts of folding or breaking are common. When the same batter is cooked on a waffle iron, the product is much less likely to have folded artifacts. The same principles strengthen a collagen membrane. We produce membranes with a reinforcing waffle configuration (Fig. 5), which is strong

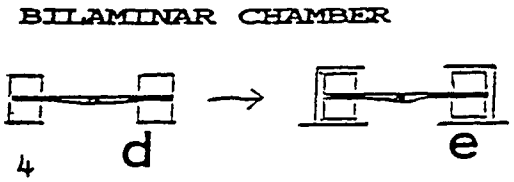
enough to manipulate in preparing waffle radial gradient chambers and several planar culture configurations. Some of the potential applications of the waffle configuration, with its myriad of geometrically patterned microcrypts, are described below.

ABNORMALITIES OF EPITHELIAL TISSUES THAT MAY BE EXPLORED IN HISTOPHYSIOLOGIC GRADIENT CULTURE

Reconstituting Essential Structures of Human Stratified Epithelium for Subsequent Experimentation

In our experience normal rat bladder, human bladder cancer removed at surgery, human bladder cancer cell line RT4, rat bladder cancer NBT-II, and chick embryo skin all proliferate in histophyiologic gradient culture and establish an organoid stratified epithelium [Leighton et al., 1984a, 1990; Leighton, 1991, 1992a,b]. A reasonable expectation is that human keratinocytes, in the presence of nutrients that support their survival and proliferation, will also establish organoid stratified epithelium with gradients of maturation in histophysiologic gradient culture. With a base line of experience established, investigations can be undertaken modifying the composition of the medium with various substances at physiological levels and in concentrations that the normal human or animal organism would find intolerable. From the point of view of clinical dermatopathology, prospective studies can be initiated in which the responses of skin neoplasia to biologic test reagents in culture may be correlated with the eventual clinical course of disease in the patient.





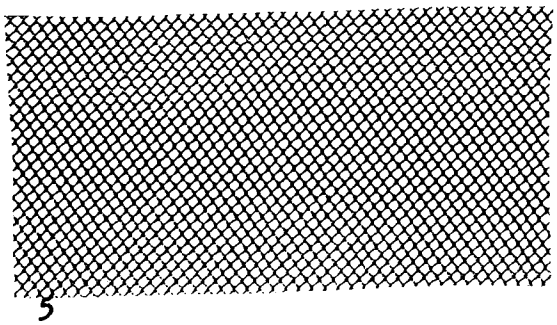


Fig. 4. Schematic drawings of unilaminar and bilaminar histophysiologic gradient cultures. **a:** A ring of cellulose sponge as a shallow cylinder, viewed as a cross section of the cylinder. **b:** A collagen membrane has been cemented to one end of the cylinder, and a tissue explant has been attached to the membrane with a thin chick plasma clot. **c:** The cylinder has been inverted so that the collagen membrane becomes the roof of the chamber. The chamber has been placed in a plastic capsule. A single aperture in the top of the capsule is the only opening for diffusion of metabolites through the collagen membrane. The capsule is placed in a large Leighton tube, and medium covers the chamber. The tube is held in a rack on a rocking platform in an incubator, and with each oscillation the collagen membrane is exposed alternately to liquid medium and to the atmosphere in the tube. **d:** When the bilaminar chamber is used, two collagen membranes are in contact, sandwiching the explanted tissue between the membranes. **e:** The ensemble is placed in a plastic capsule that has a perforation in its floor. Medium bathes both collagen membranes. The membrane near the upper opening in the chamber has a better exchange of metabolites with the medium in the culture tube.

Fig. 5. Imprint of a nylon screen, actual size. The screen is used as a template in the preparation of a collagen waffle culture membrane. There are about 45 crypts for cell implantation in each square centimeter of the collagen waffle membrane.

Creation of Interepithelial Tissue Boundaries

Since almost no experimental data on this topic are available, except for studies in developmental biology, a vast area for study is at hand. Various confrontations can be prepared using unilaminar histophysiologic gradient culture to observe the meeting of growing fronts of different kinds of epithelia, normal or neoplastic. Pairs of explants may be placed so that they are

separated by a zone of 3 to 5 mm of unoccupied collagen surface. Cells from each can migrate on the bare collagen and meet on this novel playing field.

The feasibility of this approach was confirmed when MDCK and NBT-II, as explants of packed cells, were placed on the collagen floor of unilaminar chambers. They were positioned with a separating gap of 3 mm and cemented in place with a thin chick plasma clot. The chambers were then inverted and placed in the plastic capsule. As proliferation and migration took place the gap progressively narrowed and eventually was obliterated. The MDCK grew as a translucent sheet of contiguous cells. NBT-II produced a dense population that was opaque. Histologic sections were prepared at right angle to the line of contact between the two populations. NBT-II adhered to the collagen and progressively undermined the attachment of MDCK to the membrane [Leighton et al., 1984].

To be learned through comprehensive study is whether defined boundaries occur as the migrating sheets of different kinds of cells meet, whether established boundaries are stable or move with time, and whether the movement of the boundary responds to changes in the composition of the medium.

Behavior of Pagetoid Configurations

Pagetoid patterns of invasion may be seen where normal epithelium and neoplastic inocula do not establish an interepithelial tissue boundary, but instead present a picture of individual single or groups of neoplastic cells infiltrating stratified epithelium in a diffuse pattern. The kinetics of such combinations may be observed and manipulated in studies with isotopically prelabelled cancer cells. I have searched for reference to any study of Pagetoid patterns in tissue culture without success. Medline has no references on this topic. This is an exciting opportunity for novel investigation.

Replication of Aggregates

The replication of aggregates of carcinoma cells can be observed, especially in a radial gradient setting (unpublished data). The diameter of individual aggregates can be measured on histologic section; perhaps flow cytometry applied to the contents of the chamber may provide more precise data. The cell line MDCK produces a plethora of aggregates in the chamber. Perhaps the same will result with cultures of effusion tumors. If modulating the composition of the medium facilitates the predominance of large aggregates or of small ones at the will of the investigator, the response to cytotoxic chemicals of the two categories of aggregates may be found to differ.

Aging of Cells

The aging of cells such as urothelium can be investigated in elongated radial gradient culture chambers [Leighton, 1992a]. Let us speculate. Imagine a radial gradient chamber 10 to 20 cm long. The chamber is inoculated at one end with fragments of young adult rat bladder. If proliferation, stratification, and migration are studied in this chamber for several months, cells can be expected to progressively extend along the length of the chamber for several centimeters. Cells at the growing front of the proliferation will have undergone many more mitoses during the period in culture than those remaining near the inoculum. In effect these migrating cells will be further along toward senescence than those cells remaining in the initial zone of implantation. Cells in the growing front will have had more occasions for mitotic abnormalities in their histories, either spontaneous or induced by mutagenic encounters.

HISTOLOGIC CHARACTERIZATION OF IMMORTAL CELL LINES

Catalogues of available cell lines from human or animal tumors provide a wealth of information about each line. The source, suitable medium for its propagation, genetic character, suitability for study of certain defined viruses, and often other facts are clearly provided. Absent, or almost absent, are any identifying morphologic features. At best we learn that in planar culture the growth patterns are epithelial or fibroblastic. Epithelia in nature have much more defined morphologic/histologic characteristics. Methods of three-dimensional histotypic culture are more than 40 years old. It is possible to provide investigators studying catalogues of available cells with statements and perhaps photomicrographic illustrations of the tissue these cells form in three dimensions in the absence of a participating living stroma. An optimal method of study may use a histophysiologic arrangement, perhaps using a collagen waffle membrane. A refinement of morphologic information would include references to modulations in tissue organization in response to changes in the

composition of the medium, as has been reported for rat bladder cancer cell line NBT-II in response to retinyl palmitate [see Fig. 1 in Leighton, 1992a].

TRANSFORMATION AND CARCINOGENESIS

Consider the possibility of seeding a flat collagen waffle membrane with a suspension of putative diploid cells, perhaps keratinocytes, amnion cells, or cells of the pancreatic ducts. When the cells have settled and attached at the base of most of the crypts of the waffle, the waffle can be inverted, thereby establishing a diffusion gradient in each of the isolated crypts. Foci of transformed cells may appear eventually as individual opaque crypts containing dense populations of cells, sites of transformations readily recognized with the inverted microscope. When chemicals that are known to induce transformation are applied, we may expect more countable foci of transformation.

The waffle may also serve in the assay of the histologic heterogeneity of clinical tumors. When suspensions of cells from a tumor are inoculated over the waffle, many cells will fall to the base of the crypts of collagen, attach, and proliferate. After attachment is complete the waffle may be gently rinsed to remove most unattached cells, and then inverted over a gel, producing many small histophysiologic gradient chambers. The morphologic heterogeneity of the components that are viable in culture will be evident. Studies of tumors in this model prior to clinical therapy and after recurrence will be useful. If the waffle chambers with their different colonies of cells will tolerate freeze preservation, the potential for future study will be amplified.

AN INSTRUMENT FOR TOXICOLOGIC STUDY

There is a need in toxicology testing for in vitro methods to determine the ability of toxic substances to damage living entities, and the ability for these injured entities to recover when the toxic substance is removed. Organoid groups of cells in collagen waffle crypts may be useful creatures. At some concentrations toxic agents may be lethal. At lower concentrations they may disrupt organoid associations of cells as a prelethal effect. On removal of the toxic material the organoid structures and viability may be re-established. Collagen waffle membranes in planar culture should be adaptable to the design of such assay systems.

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