

## Approach to an Organo-Typical Environment for Cultured Cells and Tissues

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*BioTechniques* 20:498-501 (March 1996)

### ABSTRACT

*If cells or tissues are taken out of an organ and put in culture, normally they lose morphological, physiological and biochemical features. This dedifferentiation process starts during the isolation procedure and continues during the whole culture period. It is caused by the stagnant liquid condition and the inadequate anchorage of cells at the bottom of tissue culture plasticware. The use of filters as basement membrane substitutes and the coating of culture-ware with extracellular matrix proteins improve the environmental factors for cultured cells but do not consider the paracrine influence of cytokines or the nutritional needs of individual cell types. To limit cellular dedifferentiation in culture, we constructed a new system, which adapts, as far as possible, cell and tissue cultures to an organo-typical environment. The system is based on a compatible cell carrier arrangement, which allows individual selection of supports for optimal cell anchorage and differentiation. The cell carriers are placed in a newly constructed container, which is permanently perfused with fresh culture medium. The system runs outside an incubator with simple laboratory tools; only a peristaltic pump, a warming table and pH-stabilized media are necessary. Without any subculturing, acute and chronic influences of drugs or the quality of medical implantation grafts can be studied over months.*

### INTRODUCTION

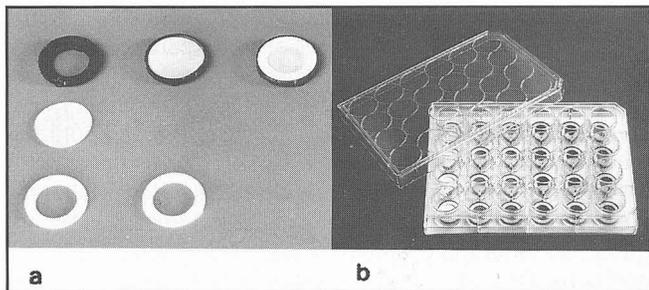
Cell and tissue cultures are indispensable tools in biomedicine and biotechnology (5). For the most part, cells are cultured with the aim of obtaining as quickly as possible a sufficient number of cells to find answers to the synthesis of a single protein or to find out the trigger mechanisms for the regulation of a channel (17). Thus the efficiency of the culture system is defined according to the cell amount and to a single

function such as the expression of a protein. Regarding the huge variety of available cell-culture models and the broad volume-scale bar ranging between a few microliters in a cloning plate and thousands of liters in a bioreactor, the inherent technical difficulties in this category of cultures seem to be solved, and the problem of cellular dedifferentiation can be neglected (4,5).

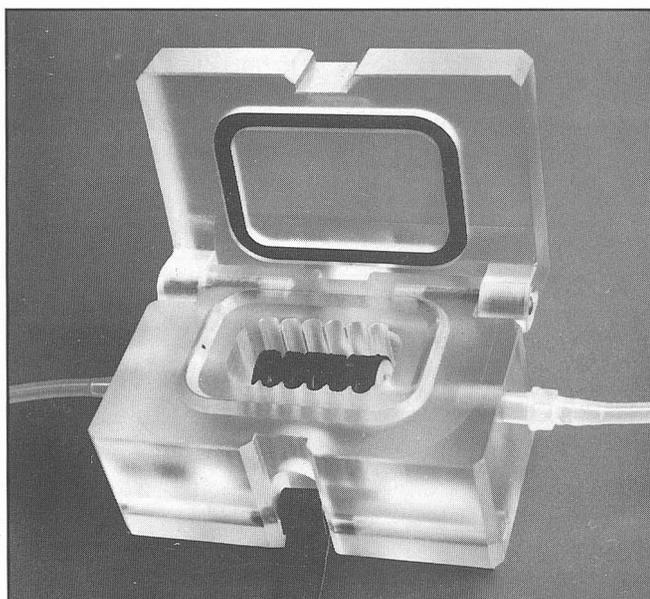
Besides the bulk-production, cells or tissues are isolated and brought in culture to study specific organ functions and complex tissue interactions (15). In such organoid models, the cultured cells from liver, kidney, lung and brain should exhibit an original functional phenotype to study the influence of pharmaceutical substances out of an organism but close to the natural situation (6). The same quality of cultures is requested for testing surgical implantation grafts to evaluate the biocompatibility of new materials for artificial articulations, blood vessels or dental prostheses (5). Standard for the cultures should be the immediate transfer of data from the culture experiments to the situation within an organism. Thus, the cultures have to retain almost the same characteristics, which are found in the organ or tissue from which they derived. However, numerous investigations have shown that typical functions of cells and tissues are lost during the isolation and subsequent culture (8). This uncalculable dedifferentiation process is caused by an insufficient anchorage of cells, by suboptimal nutrition and by an unbalanced environment of cytokines within the stagnant liquid conditions of tissue-culture plasticware (6). The use of different kinds of filters as artificial cell supports or the coating of tissue-culture plasticware with extracellular matrix components resulted in an improved quality of cultured cells (3,18,19). This knowledge was the base to elaborate an advanced culture system to mimic an organo-typical situation for further improvement of cell differentiation during culture (9,10).

## MATERIALS AND METHODS

The principle of the method is based on the fact that the anchorage influences the differentiation of cultured cells (19). For that reason we work with individual supports, for example, different kinds of filters, which are mounted in a flat cell carrier arrangement (Figure 1a). The so-called MINUSHEET (MINUCELLS and MINUTISSUE; GmbH, Bad Abbach, Germany) is placed at the bottom of a culture dish (Figure 1b). The cells are pipetted onto the individual filters, where they spread out. It is also possible to mount thin tissue slices in the cell carrier arrangement, which are placed in a sandwich manner between two nylon nets. For optimal nutrition, the cell carriers are transferred, using forceps, to the new perfusion container (Figure 2). A slowly rotating peristaltic pump renews the medium, and a warming plate produces the right temperature (Figure 3). Because HEPES-buffered, Leibowitz or other pH-stabilized media are used, it is possible to



**Figure 1. Cell carriers allow an individual selection of supports for optimal anchorage and differentiation of cultured cells.** a) The cell carrier consists of 3 parts: a black holder ring, the selected support with 13-mm diameter and the white span ring. For mounting, the support material is placed into the holder ring, then the white span ring is pressed down. b) After autoclaving, the cell carrier is placed into a 24-well tissue-culture plate. For adhesion, the cells are transferred within a droplet of medium onto the support.



**Figure 2. Perfusion culture container.** After adherence of cells on the support, the cell carriers are transferred, using a fine forceps, to a newly constructed perfusion culture container. Medium is pumped in at the left side, crosses between the cell carriers and leaves at the right side.

run the system out of the atmosphere of a CO<sub>2</sub>-incubator on a laboratory table.

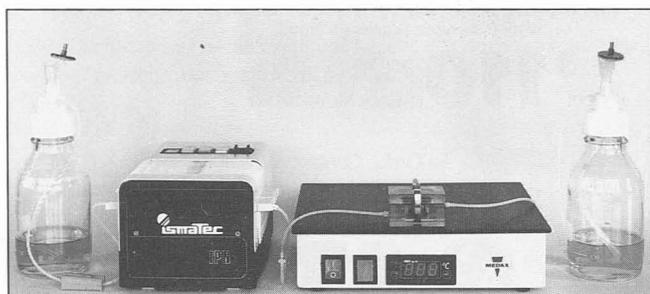
To elaborate, the optimal support for the cultured cell type, the flat cell carrier is used (Figure 1a). It is made of MACRALON® (Hoechst, Offenbach, Germany) and consists of a black holder ring in which an individually selected support material for optimal cell anchorage is placed. A variety of commercially available supports, like glass, foils, filters and nets with 13-mm and 47-mm diameters, fit in the cell carriers. Excised biological materials such as the fibrous capsule of the kidney also serve as excellent supports (13). To keep the individual support in position, the white spann ring is pressed into the black holder ring. Then the cell carriers can be autoclaved or sterilized in liquids such as ethanol or formaline, depending on the support materials used. After sterilization, the cell carriers are placed, using a fine forceps, in a culture dish (Figure 1b). The cells are transferred within a droplet of medium to the surface of the support. The cells adhere and spread out. If transparent supports are used within the cell carriers, the growth of cells is controlled within the culture dish by an inverse microscope equipped with phase-contrast optics. Cells attached to nontransparent supports are fixed for optical control within the cell carriers in 70% ethanol, stained, for example, with propidium iodine or a fluorescent antibody. Then the cell carrier is turned upside down, and the cells are visualized with an inverse microscope equipped with epifluorescence optics (13).

Differentiated cells need optimal nutrition, a continuous elimination of harmful metabolic products and a stabilization of the synthesis of cytokines, which influence a variety of cellular functions (2,18). Because a consideration of all these factors is impossible under the stagnant conditions in the tissue-culture plasticware, a perfusion container made of polycarbonate was used (Figure 2). The cell carriers with adjacent cells are placed, using a forceps, in the container. Over silicone tubes, Luer-Lok® fittings and specific screw caps, the container is connected with two media bottles (Figure 3) so that the whole working line is autoclavable (110°C, 0.5 bar). A peristaltic pump transports the medium into the container, where it crosses between the cell carriers and flows out at the upper side. Using a container for 6- × 13-mm cell carriers (Figure 2) and a perfusion of 1 mL/h over 14 days of Iscove's modified Dulbecco's medium (Life Technologies, Eggenstein, Germany), with or without 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany) and 1 × 10<sup>-6</sup> M Aldosterone (Sigma-Aldrich Chemie GmbH, Deisen-

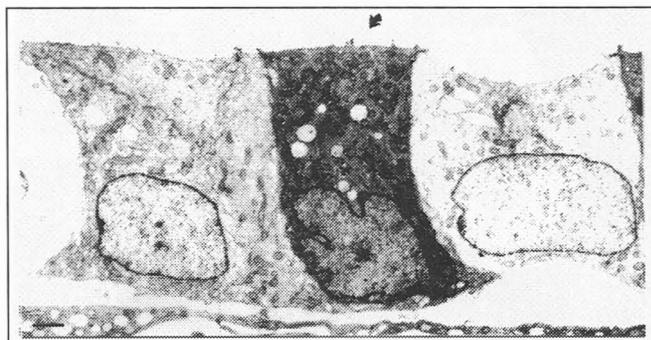
hofen, Germany) resulted in an optimal differentiation of tubular (1,11,12) and endothelial (7) cells from the kidney. Sittinger et al. used a stop-and-go perfusion with higher rates than 1 mL/h for the generation of cartilage tissue (16). Since the cells stay in "contact inhibition" during perfusion culture, the mitotic activity is low in these experiments and thus adapted to the natural situation.

## RESULTS AND DISCUSSION

For many years it was known that perfusion of media (14), selection of a basement membrane substitute (3,19) and adaptation to the natural environment improve the quality of cultured cells (6). On the basis of this, we developed a handy new culture system (9–13). We observed that embryonic renal collecting duct cells differentiated in perfusion culture to a heterogeneously composed epithelium comparable to the tissue found within the adult kidney (1,11,12) (Figure 4). As known from the kidney, the epithelial cells are "contact inhibited" and therefore can be maintained over a period of months or longer without subculturing. Earlier experiments under stagnant culture conditions failed. Subsequently, for the first time it was shown by immunohistochemistry that embryonic endothelial cells from the renal microvasculature could be maintained over a period of several weeks in perfusion culture (7). The cells synthesized a newly detected endothelial antigen (EnPo I) and formed a meshwork of capillaries as found within the embryonic kidney. Under conventional culture conditions, the endothelial precursor cells degenerated. Last but not least, chondrocytes cultured on bioresorbable fleeces and under perfusion culture mantled themselves by extracellular matrix and synthesized collagen fibrils in their close vicinity (16). No switch in natural collagen Type II synthesis was observed. Thus, chondrocytes cultured under perfusion conditions acquired morphological and cell biological properties comparable to those displayed by cartilage tissue. Experiments under stagnant culture conditions failed. Summing up the recent results, we demonstrated that the quality of cultured cells and tissues can be improved greatly, if an optimal support for the cells is chosen, if permanent subculturing is omitted and if the cells are perfused for the whole culture period with fresh medium. Thus, mimicking the natural situation, it helps to solve the problem of cellular dedifferentiation in culture.



**Figure 3.** The perfusion culture system runs on a laboratory table. The medium is transported with a peristaltic pump with 1 mL/h from the left bottle into the container on the warming plate (37°C). The waste medium is collected in the bottle at the right side. Because pH-stabilized media are used, the system works outside an incubator for weeks and months.



**Figure 4.** Renal collecting duct epithelium after 14 days of perfusion culture conditions. Transmission electron microscopy shows a collecting duct epithelium consisting of a dark, mitochondria-rich intercalated cell (arrow) between light principal cells (bar = 1 µm).

## ACKNOWLEDGMENTS

The investigation was supported by the Deutsche Forschungsgemeinschaft (Mi 331/4-1). The skillful technical assistance of Marion Kubitz is gratefully acknowledged. All parts of the described culture system are available by MINUCELLS and MINUTISSUE Vertriebs GmbH, Starenstr. 2, D - 93077 Bad Abbach, Germany.

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