The use of a conventional tissue culture plate as an optically accessible perfusion chamber for *in situ* assays and for long-term cultivation of mammalian cells

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Abstract

An alternative culture system has been developed based on a conventional tissue culture plate (3.5 cm diameter) which is changed into a closed perfusion chamber. The system can easily be scaled up from one to several chambers. The shape and the size of the area of cell growth may be designed to individual experimental demands. The whole culture chamber is optically accessible, so cell growth and morphology can be evaluated by light microscopy. Furthermore the cellular physiology can be characterised by any fluorimetric assay using a bottom type fluorescence reader. A peristaltic pump sustains a constant medium flow through the chamber thus creating true homeostasis. The use of HPLC-valves and connectors allows the switching between different media or assay solutions. Thus it is possible to perform *in situ* assays also measuring transient effects. A protocol for vitality tests using calcein-AM is worked out for an adherent cell line and for a suspension cell line. The lower detection limits are 7×10^2 cells cm^{-2} for the adherent cells and 5×10^4 cells mL^{-1} for the suspension cells. The upper limits are $1-2 \times 10^5$ cells cm^{-2} respectively 8×10^6 cells mL^{-1} .

Abbreviations: FGF – fibroblast growth factor, HUVEC – vascular endothelial cells from human umbilical cord veins, IF – 1:1 mixture of Iscove's MDM and F12 basal media, NCS – newborn calf serum, PBS – phosphate buffered saline, TE – 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS

Introduction

Mammalian cells originate from a vascularized tissue or from a body fluid with a controlled environment. *In vitro* cells are usually grown in artificial culture media, in tissue culture flasks and in a batch mode and thus are exposed to an unphysiological situation. One characteristic shortcoming of such a treatment is the lack of homeostasis with temporary gradients of for example metabolic waste products, growth factors and nutrients (Glacken 1988). So *in vitro* many primary cells exhibit an abnormal physiology leading to dedifferentiation (Koechlin et al., 1991; Nerem et al., 1993) or an altered metabolism (Schrimpf et al., 1994; Nollert et al., 1991; Brand et al., 1994).

An improved culture system should provide the cells with an environment of constant composition (Singhui et al., 1994). In principle this can be simply achieved by perfusion of the culture volume as for example by running a bioreactor in perfusion mode. The problem is the scale-down of this technique to the size of a small flask or plate culture. Some systems have been described with special small size containers and/or cells growing on special filter supports (for example Minuth et al., 1992; Munn et al., 1994; Usami et al., 1993). However this means that the routines of cultivation have to be changed in some important points: the cells have to switch from the usual tissue culture plastique to the new support material; the microscopic examination of the culture is impossible

Figure 1. Assembled perfusion chamber and silicone sheet geometry. Cells are cultured in a tissue culture plate with a silicone sheet (0). After removal of sheet (0) the first and second silicone sheet are laid onto the surface of the plate. A polycarbonate inlet is placed on top of the two sheets. For cultivation of suspension cells a polycarbonate membrane is stretched between the two silicone sheets. The medium flow is sustained from a peristaltic pump through the inlet and outlet of the perfusion chamber. The geometry of the chamber is committed from the holes in the silicone sheet.

Figure 2. Operation of the perfusion system. The perfusion chamber is mounted on a aluminium frame and is placed on a heating plate (37 °C). The medium reservoir is kept at 4 °C and a peristaltic pump transports it to a water bath (37 °C) with a heating tube. A HPLC injection valve is switched in front of the perfusion chamber for injection of fluorescent probes. The used medium is gathered in a waste reservoir. The aluminium frame with the perfusion chamber is moveable and can be placed under a fluorescence microscope or in a fluorescence reader.

or hindered severely.

Our aim is the development of a system based on a conventional tissue culture plate, so that no adaptation is necessary. The system shall be optically accessible: at any point of cultivation the culture can be examined by light microscopy without opening the system and removal of covers, filter supports etc. so that the cultivation can be continued as easily as with a culture flask being put back into the incubator. A further advantage of such a system is the spectroscopical access from beneath due to the high optical quality of tissue culture plastic.

The combination of a freely positionable bottom type fluorescence reader such as the Fluostar™ (BMG LabTechnologies, Offenburg) with the above described culture system will allow the establishment of a fluorimetric microphysiometer by use of the great variety of fluorescent probes for cellular physiology. Here we describe the modification of a tissue culture plate (35 mm diameter) to a perfusion chamber and some examples for studying cellular physiology *in situ* by fluorescence analysis.

Material and methods

Materials

Basal tissue culture medium, a 1:1 mixture of Ham's F12 and Iscove's DME (IF) is purchased from GIBCO, newborn calf serum (NCS) from Sebak (München), trypsin '1:200' from Serva (Heidelberg), gelatin and heparin from Sigma (München), transferrin from Behring (Marburg); Calcein-AM from Molecular Probes Europe (Leiden), all other chemicals are supplied by Merck (Darmstadt). Tissue culture flasks and plates are purchased from Greiner (Frickenhausen), HPLC components from GAT (Bremerhaven). Fibroblast growth factor (FGF) is applied as a crude partially purified preparation from porcine brains as previously described (Friedl et al., 1989; Gospodarowicz et al., 1978).

Cell culture

HUVEC are isolated from umbilical cord veins by a modification of the previously published method of Gimbrone et al. (1978) and maintained in culture as described (Friedl et al., 1989). The endothelial cells are subcultured by treatment with trypsin-EDTA and seeded into gelatin-coated tissue culture substrates at

Figure 3. Fluorescence distribution in the perfusion chamber. A fluorescein-PBS solution is pumped through the perfusion chamber. After entrance of the fluorescence indicator the area is scanned like indicated with crosses. 5 and 10 min later further scans are done.

Fluorescence course : Fluorescein [$10\mu\text{M}$], duration 4min

Two 0.25mm silicone sheets

Two 0.25mm silicone sheets with polycarbonate membrane

Figure 4. Fluorescence course with and without membrane. Fluorescence measurements in the center of the perfusion chamber during a fluorescein impulse (4 min). Every 2 seconds a fluorescence value is taken and the replacement of the indicator is pursued. The time for a fluorescence wave is almost doubled for chamber use with polycarbonate membrane.

Protocol for fluorescence measurement with Calcein-AM

Figure 5. Vitality test kinetics with Calcein-AM and EA.hy926 cells. The vitality fluorescence indicator Calcein-AM is perfused for 30 min into the chamber. Calcein-AM is diluted in perfusion medium without serum (because of esterase activity). Then the injection valve is switched to perfusion medium. The cells need further 30 min to process the non-fluorescent indicator. The fluorescence signal corresponds to the vitality of the cultured cells.

a split ratio of 1:3 up to 1:5. The culture medium consists of basal medium IF plus 15% (v/v) NCS, 2 mM glutamine, 20 $\mu\text{g mL}^{-1}$ heparin, 5 $\mu\text{g mL}^{-1}$ iron-saturated transferrin and 0.35 $\mu\text{g mL}^{-1}$ crude porcine FGF. Ea.hy926 cells are kindly provided by Dr. Edgell et al. (1983). The medium consists of basal medium IF plus 10% (v/v) NCS. The cells are cultured at 37 °C in 5% CO₂.

For use in the perfusion system the media are modified: 25 mM HEPES is substituted for the usual NaHCO₃; NaCl is added for maintaining the osmotic balance.

Results and discussion

Assembly of the perfusion chamber

A conventional tissue culture plate of 35 mm diameter

is used as the bottom part of the perfusion chamber. The cells are preincubated with a silicone sheet (0) (1 mm thick, 11.3 mm diameter) that is laid in the tissue plate before cultivation (Figure 1). A hole of defined geometry (Figure 1) is punched into another silicone sheet (0.125–0.5 mm thick) and the sheet placed onto the surface of the tissue culture plate. A second silicone sheet with a slightly prolonged hole (Figure 1) is prepared and put on top of the first sheet; in case of only weakly adhering cells or suspension cells a perforated polycarbonate membrane (Whatman) is placed between the two sheets for retention of the cells in the lower compartment of the chamber. A cylindrical inlet (34.5 mm diameter, 18 mm height) of polycarbonate is placed on top of the two silicone sheets (Figure 1). The inlet has a center hole (15 mm diameter), so that above the area of cell growth the polycarbonate has a thickness of only 1 mm. This allows the microscopical examination of the culture by phase contrast microscopy with

Correlation between adherent cell number and fluorescence value

Figure 6. Correlation between adherent cell number and fluorescence. A defined cell number is seeded into 35 mm tissue culture plates and after 2 h the perfusion system is assembled. The test is performed with 10 μ M Calcein-AM and the fluorescence measurement (gain settings: 40 for $2 \times 10^5 - 5 \times 10^4$ cells cm^{-2} ; 50 for $2 \times 10^4 - 1 \times 10^4$ cells cm^{-2} ; 60 for $5 \times 10^3 - 2.5 \times 10^3$ cells cm^{-2} ; 70 for $1.25 \times 10^3 - 7 \times 10^2$ cells cm^{-2}) is done after 30 min loading and 30 min lag-time. The lower detection limit is 7×10^2 cells cm^{-2} . A linear regression covers the range up to confluence.

an inverse optic. The chamber is sealed by an O-ring (1.5 mm thick, 32 mm diameter) resting in a groove on the side wall of the inlet (Figure 1). Two opposing vertical channels (6 mm diameter) are bored from the top to the bottom of the inlet separated by a distance of 25 mm. At the outer surface the channels are modified for the uptake of a Peek Fitting (GAT). The inlet is positioned on top of the upper silicone sheet so that the opening of the channels is centered with respect to the height of the sheet's slit. One channel is the port for incoming medium and is connected via a peek or silicone tubing to the marprene tubing of a peristaltic pump (Watson Marlow), delivering medium from a reservoir (Figure 2). The other channel is the port for medium outlet and is connected via peek/silicone tubing with a waste reservoir (Figure 2). The perfusion chamber plus the tubings are assembled in a sterile hood and can then be used outside.

Operation of the perfusion system

The chamber is mounted on one place of an aluminium frame with the dimension of a 6-well plate; each single place has a bottom hole located at the area of cell growth. The frame is put on a heating plate and the temperature set at 37 °C (Figure 2). The medium reservoir is kept at 4 °C. The peristaltic pump transports the medium through a bubble trap consisting of a 10 mL tube heated at 37 °C into the perfusion chamber. The pump rate is adjusted between 10–1000 $\mu\text{L min}^{-1}$. This leads to laminar streaming conditions and the shear stress level for the cultured cells behave between 0.001–1 dyn (DePaola et al., 1992). In the cell culture media 25 mM HEPES is substituted for the carbonate buffering; NaCl is added for osmotic balancing.

An HPLC injection valve with an appropriate sample loop is located between the bubble trap and the

Correlation between suspension cell number and fluorescence value

Figure 7. Correlation between suspension cell number and fluorescence. After cell counting cells are seeded into a culture plate with one silicone sheet. The polycarbonate membrane and the second sheet is laid on top and the inlet closes the chamber. The test is performed with $10 \mu\text{M}$ Calcein-AM and the fluorescence measurement (gain settings: 25 for 8×10^6 cells mL^{-1} ; 35 for 4×10^6 – 2×10^6 cells mL^{-1} ; 45 for 1×10^6 – 5×10^5 cells mL^{-1} ; 55 for 2×10^5 – 1×10^5 cells mL^{-1} ; 65 for 5×10^4 cells mL^{-1}) is done after 60 min loading time and 30 min lag-time. The lower detection limit is 5×10^4 cells mL^{-1} this corresponds to 1×10^4 cells in the perfusion chamber. The upper correlated cell number is 8×10^6 cells mL^{-1} .

perfusion chamber (Figure 2). The sample loop can be loaded with a syringe containing either a fluorescent probe or a biologically active mediator. A sterile filter is placed between the syringe port and the sample loop. A 4-way-valve can be mounted alternatively/additionally between medium reservoir and pump for special purposes. Usually there is a large excess of culture medium to cell number: for example a 15 mL tube filled with medium; in routine flask culture the ratio of volume per surface is about 0.2 mL cm^{-2} . In such a case the medium can be circulated from the medium reservoir through the chamber back to the medium reservoir for a certain time without severe impairment of homeostasis.

Gradients along the chamber length

Along the chamber length there will be constant gradients of waste products, nutrients and growth factors due to the consumption/production of these components by the cells. However *in vivo* these gradients exist in blood vessels and the surrounding tissue too. The problem is solved by a sufficient flux rate. Although the growth area is not rectangular (the vertical sides are no straight lines but half circles), the gradient of a component is calculated on the basis of a perfect rectangular geometry for reasons of simplicity. This will lead to a slight underestimation of the concentrations of components to be consumed respectively to a slight overestimation of the concentrations of components to be produced. So in both cases limiting concentrations calculated this way will be reliable.

A volume element containing the initial concentration c_o of a component x is moved along the chamber length with a velocity determined by the pump rate p [$\mu\text{L min}^{-1}$] and the geometry of the chamber volume – length l , height h and width b [mm]. The distance which has been passed by the volume element can thus be calculated from the time t [min] as

$$l = \frac{t \cdot p}{b \cdot h} \quad (1)$$

The concentration of a component has been changed at this distance by

$$c_l = c_o + r_x \cdot t \quad (2)$$

with r_x being the volumetric reaction rate of the component x [moles $\text{min}^{-1} \mu\text{L}$] – negative for consumption and positive for production rates. The parameter t is substituted from (1) by l

$$c_l = c_o + \left[\frac{r_x \cdot b \cdot h}{p} \right] \quad (3)$$

The volumetric reaction rate of the component x can be calculated using measured or published values for the specific cellular consumption rate s_x [moles $\text{min}^{-1} \text{cell}^{-1}$], the cell density d [cell mm^{-2}] and the chamber height h [mm]

$$r_s = s_x \cdot \frac{d}{h} \quad (4)$$

with r_x being the volumetric reaction rate of the

The concentration of a component is thus given by

$$c_l = c_o + \left[\frac{s_x \cdot d \cdot b}{p} \right] \cdot l \quad (5)$$

Using Equation (5) the concentration of several nutrients at the chamber outlet can be calculated for example for several endothelial cells and cell lines. It shows that oxygen is the first component to become limiting. For HUVEC – a cell type with an extremely high oxygen demand (Kjellstrom et al., 1987; Friedl et al., 1989) – the specific cellular oxygen uptake rate is $2.86 \times 10^{-9} \text{ mg O}_2 \text{ cell}^{-1} \text{ h}^{-1}$ and the minimum pump rate has to be $5 \mu\text{L min}^{-1}$ for maintaining the air saturation above 70% in the whole perfusion area.

Performance of the perfusion system

PBS is pumped through the chamber (two 0.25 mm sheets, $56 \mu\text{L}$ volume) at a rate of $10 \mu\text{L min}^{-1}$ and suddenly substituted by a fluorescein-PBS solution via

a injection valve. At the indicated times the growth area of the chamber is scanned by the FluostarTM. Figure 3 demonstrates that there was a uniform flow of liquid through the chamber. At the chosen pumping rate it takes 9 min for a complete replacement of the chamber content. A similar experiment (two 0.25 mm sheets, $56 \mu\text{L}$ volume) is performed at another pump rate of $120 \mu\text{L min}^{-1}$ with and without a separating polycarbonate membrane between the two silicone sheets (Figure 4). Only the center point of the chamber is scanned every two seconds to monitor the kinetics of fluorescence influx. The period necessary for medium exchange is prolonged from 1 min to 2–3 min due to the inserted polycarbonate membrane.

Figure 5 shows a vitality test of confluent EA.hy926 cells. At the indicated time the medium is switched to medium containing the membrane-permeable non-fluorescent calcein-AM indicator ($10 \mu\text{M}$) for 30 min. The calcein-AM perfusion medium has no serum portion because of esterase activity in NCS. During this loading time calcein-AM enters the cell via diffusion and cellular esterases hydrolyze the calcein-AM into the membrane-impermeable fluorescent calcein and acetate. The release of calcein from calcein-AM requires the hydrolytic cleavage of 7 ester bonds and seems overall to be a slow process as the fluorescence still increases after the medium has been switched back to blank medium. So for the other experiments with calcein-AM we choose a lag-time of 30 min before measuring the fluorescence.

In another experiment EA.hy926 cells are seeded at the indicated cell densities in 35 mm tissue culture plates and cultivated in an incubator for 2 h to allow cell attachment. Then the perfusion system is assembled and a vitality test performed as described above ($10 \mu\text{M}$ calcein-AM, $56 \mu\text{L}$ chamber volume, a pump rate of $120 \mu\text{L min}^{-1}$, 30 min loading, 30 min lag-time). Figure 6 presents the correlation between number of seeded cells and fluorescence. Neglecting the increase in cell number for the spent time between seeding and assay (2–3 h) and the decrease in cell number by an attachment efficiency < 1 the lower detection limit is at $7 \times 10^2 \text{ cells cm}^{-2}$ and the assay covers the range up to confluence (Figure 6). This range can only be covered by stepwise changing the gain factor of the photomultiplier from 40 for $2 \times 10^5 \text{ cm}^{-2}$ to 70 for $7 \times 10^2 \text{ cells cm}^{-2}$. Increasing the gain by 10 increases the sensitivity twofold.

The same experiment is done with PAI-5 myeloma as example for suspension cells. Figure 7 shows the correlation of fluorescence with cell number with

Figure 8. Phase contrast pictures from cultured HUVEC cells. a) Primarily isolated HUVEC, passage number 2, 3 days in culture (x20). b) Primarily isolated HUVEC, passage number 2, after 14 days confluent culture (x20). c) Primarily isolated HUVEC, passage number 2, after 14 days confluent culture in the perfusion system. (0.1 dyn shear stress; x40).

the standardized protocol for suspension cells (10 μM calcein-AM, 56 μL chamber volume, a rate of 120 $\mu\text{L min}^{-1}$, 60 min loading, 30 min lag-time). The loading time is prolonged due to the slower displacement of the lower chamber content beneath the membrane. The lower detection limit is $4 \times 10^4 \text{ cells mL}^{-1}$ and the upper limit is $8 \times 10^6 \text{ cells mL}^{-1}$ (Gain factor of photomultiplier from 65–25). In this case $5 \times 10^4 \text{ cells mL}^{-1}$ corresponds to 1×10^4 cells in the volume of the perfusion chamber.

Long-term cultivation is carried out with primarily isolated HUVEC. A confluent layer of HUVEC is cultivated in the perfusion system (56 μL chamber volume, a rate of 360 $\mu\text{L min}^{-1}$). For long-term cultivation the system is modified to circulation mode, so the medium is pumped from the reservoir through the chamber back to the medium reservoir. After 14 days the cells are tested for vitality (Calcein-AM) and the changes in morphology under shear conditions are shown in Figure 8. The result is a vital cell layer. The cells are aligned to the direction of the streaming medium. The parallel culture under static conditions without splitting have shown a senescent dying cell layer. The perfusion system offers the opportunity to culture endothelial cells for long-term in a more physiological way as it is possible under static conditions.

Conclusions

We have developed a perfusion system on the basis of normal cell culture plates. The cultivation of various cell lines is possible under more physiological conditions than in static culture. Furthermore adherent cells are cultured under defined shear stress conditions. The working system is accessible for fluorescence measurements and microscopical observations so the cells can be characterised by any fluorimetric assay. The wide range of fluorescence indicators allows the examination of many parameters of cell physiology under perfusion mode.

Note added in proof: meanwhile the whole system (Cytostar) as well as individual components can be purchased from BMG Lab Technologies (Offenburg, Germany, tel. +49 781 67611).

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