Cellular Engineering

On-line control of cellular adhesion with impedance measurements using interdigitated electrode structures

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Abstract—*Critical parameters to be assessed in cell culture are the number of viable cells and cell viability. Growth, product formation, toxicity effects and the overall success of cell culture can depend largely on these. With interdigitated electrode structures (IDES) adherent cells are cultured directly on a pair of interdigitated electrodes, and the impedance of the system gives insight into the adhesive behaviour of the cells. The signal is influenced by the changes in number, growth and morphological behaviour of adherently growing cells, mainly owing to the insulating effects of the cell membranes. Five different cell lines are used, and their divergent behaviour is monitored over a period of four days, from inoculation of the cells to killing of the cells at the end of the experiments. Even when the cells form close monolayers, great fluctuations in the impedance signal can be observed. Nevertheless, for a more complete description of cellular systems, other parameters, such as acidification and respiration, have to be included in the measuring system.*

Keywords—*Cell-based biosensor, Interdigitated electrode structures, Impedance, Cellular adhesion, PhysioControl-Microsystem*

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1 Introduction

TUMOUR PROGRESSION is a multistep process. Neoplastic clones are usually generated by a series of somatic and/or inherited mutations. Those genetic changes allow cells to bypass programmed cell death, avoid the need for growth factors (positive regulators), ignore restraining signals (negative regulators), escape from immunological surveillance, stimulate the formation of their own blood supply (angiogenesis), breach surrounding tissues (invasion), and often colonise distant sites (metastasis).

Malignant tumour cells generally metastasise via the lymphatic and/or vascular system. During the haematogenous phase of metastasis, tumour cells undergo extensive cell–cell and cell–extracellular matrix interactions. Therefore adhesions are essentially involved, although at different levels, in all the tumour cell–host interactions. In fact, cell adhesion is the fundamental process of all biological systems (HONN *et al.*, 1995). In addition, more than 70% of malignant tumours are of epithelial origin (HENDERSON *et al.*, 1991), and therefore adhesion is an interesting parameter.

To obtain greater insight into the mechanisms of cellular signalling, it is advantageous to detect several cellular output signals in parallel (BRAY, 1990; KRAUS and WOLF, 1995). This is a fundamental requirement for many questions in the fields of basic research and applied biomedical research (pharmaceutical analysis, applied biotechnology, biomedical material research, chemotherapy, toxicology and environmental biology).

Signal parameters such as changes in pH (intracellular or extracellular), oxygen consumption, ion concentrations, impedance of cellular systems, membrane potentials, release of proteins and metabolites can be measured by electric and/or optical sensor devices. Based on these considerations we developed a device called the PhysioControl-Microsystem (PCM) (BRISCHWEIN *et al.*, 1996; WOLF *et al.*, 1994), incorporating appropriate sensor devices and permitting high-resolution microscopy. Important sensoric components integrated into the PCM are impedance measurements with interdigitated electrode structures (IDES) (WOLF *et al.*, 1998). They allow the control of cell adhesion to the substrate.

The dielectric properties of biological materials (amino acids, polypeptides, proteins, cell suspensions, tissues) have been studied for a long time in the frequency range from 1 Hz to a few gigahertz (FOSTER and SCHWAN, 1989; PETHIG, 1979). For example, impedance techniques have been used to investigate changes in the membranes of biological cells in suspension (PLIQUETT and WUNDERLICH, 1989; MATANGUIHAN *et al.*, 1994), to measure micromotion of cells on a small electrode (GIAEVER and KEESE, 1993), and to determine transepithelial and transendothelial electrical resistances of cultured cell monolayers (WEGENER *et al.*, 1996).

Critical parameters to be assessed in cell culture are the number of viable cells and the physiological state of cells. Growth, product formation, toxic effects and the overall success of cell culture can depend on these parameters to a great extent. Viable cells are commonly measured microscopically after the cells have been suspended in a dye such as Trypan Blue.

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Another possibility is the use of a fluorescence activated cell scanning (FACS) device. To count or scan the cells, aggregated cells have to be dispersed by means of chemical and enzymatic methods. More intricate procedures have to be applied for anchorage-dependent cells. Therefore, it is advantageous to have an on-line, real-time measurement method.

With IDES, adherent cells are cultured directly on a pair of interdigitated electrodes. Impedance measurements on IDES result in an integral signal that is influenced by changes in number, growth and morphological behaviour of adherently growing cells. The measurements can be performed for several days as there is no detectable electrical influence on the cells.

The versatility of this new sensor was shown with some sample experiments (EHRET *et al.*, 1997). Cell density, growth and long-term behaviour of cells on the electrodes clearly change the impedance of the IDES. Both the global influence of serum components (deprivation of fetal calf serum) and the toxic effects of heavy metal ions (cadmium) result in changes in the sensor signal and could be visualised.

Briefly, the cellular impedance signal results from insulation by the cell membranes. If cells are placed on the electrodes, they block the current flow in a passive way, and the impedance increases.

In this study, five different cell lines were used, and their divergent behaviour was monitored over a period of four days, from the inoculation of the cells up to the killing of the cells at the end of the experiments. Significant differences in initial adhesion and further growth of the cells could be observed. Stimulations and disturbances could be noticed very early. Nevertheless, knowledge about adhesion alone is not sufficient to describe complex cellular systems. Other parameters, such as acidification and respiration, have to be included in the measuring system for a more complete analysis (WOLF *et al.*, 1998).

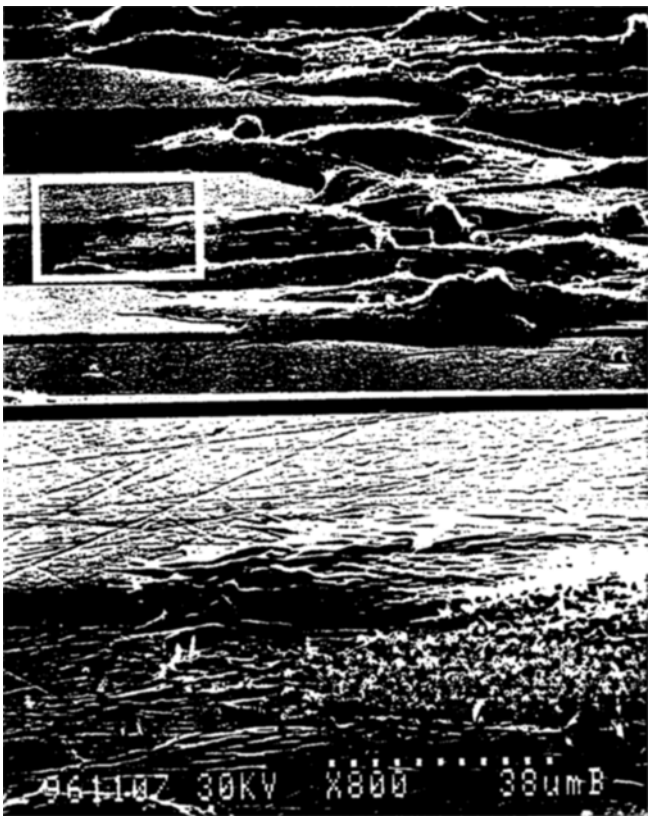

Fig. 1 *SEM micrograph of LS 174T cells on IDES. Adhesion on electrodes and substratum are clearly visible. Dotted line indicates length of 38 μm for upper half of micrograph and 7.6 μm for lower half, which is an enlargement of the rectangle in the former*

2 Material and methods

2.1 *Sensor fabrication*

The sensors were fabricated in co-operation with G. Sulz (Institut für Physikalische Meßtechnik der Fraunhofergesellschaft, Freiburg, Germany) as described elsewhere (EHRET *et al.*, 1997). The sensitive area of the IDES is $5 \times 5\,mm^2$. A trough made of epoxy resin is fixed on the IDES to allow the growing of cells on the sensitive area. The volume of the culture medium is about 200 μl. The width of the electrodes and the distance between the electrodes are both 50 μm (Figs. 1 and 2). The distance from the top of the platinum electrodes to the sapphire substrate is 400 nm. All materials in contact with cells have been intensively tested for their biocompatibility. No differences to normal culture behaviour, either cell density or morphology, could be observed.

2.2 *Cell types and culture of cells*

Five different cell lines were used for the experiments. LS 174T (ATCC CL 187) was derived from a human colon adenocarcinoma. Electron microscopic studies revealed abundant microvilli. LS 174T cells grow in an epithelial-like manner and have been well characterised in our laboratory (WOLF *et al.*, 1984). This is a necessary prerequisite for experiments at this early stage of sensor development. Several culture conditions and the cellular behaviour are well known and have been routinely examined (scanning and transmission electron microscopy, phase-contrast light microscopy, determination of cell number/density, use of dyes) to detect any differences between experimental and normal cell culture.

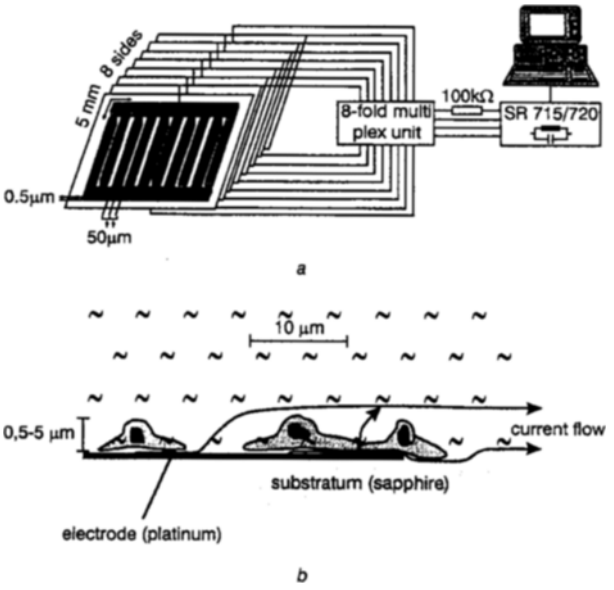

Fig. 2 *(a) Block diagram of assembly used for measurements with SR 720. 100 kΩ resistor is needed for current reduction. Multiplex unit allows measurement of eight IDES in parallel. Data acquisition is computer controlled. (b) Main effect of cells on IDES is due to insulating property of cell membrane. The presence of intact cell membranes on electrodes and their distance from electrodes determine the possible current flow and thus the sensor signal*

This refers to biocompatibility testing of the materials used as well as to the experimental conditions on the IDES. In most experiments, the LS 174T is used as a reference.

CV-1 (ATCC CCL 70) was derived from the kidney of a male adult African green monkey. The cells grow rapidly and form monolayers of fibroblast-like cells. 293 (ATCC CRL 1573) was derived from human embryonic kidney cells. It forms monolayers and grows in an epithelial-like manner, as does HeLa (ATCC CCL 2), which was derived from a human cervix adenocarcinoma. RT112 (DKFZ) was derived from a human bladder carcinoma and also grows in monolayers.

Normal culturing of the cell types used was done under standard conditions of 37°C and 5% CO_2 in air with medium consisting of Rosswell Park Memorial Institute (RPMI) medium (25 mM HEPES-buffer), supplemented with 10% fetal calf serum (FCS) and 1% non-essential amino acids. The medium was supplemented with antibiotics, except for CV-1. These cells are cultured without antibiotics. IDES were not pretreated with any adhesive support such as components of the extracellular matrix.

The number of cells to be seeded on the IDES was determined as follows: The cell culture petri dishes used had an area of 5945 mm^2. The area of an IDES was 25 mm^2. When a petri dish was completely covered with cells, it could be used for the experiments or for subculturing. This was done by use of a trypsin/EDTA-solution to detach the adherent cells, followed by washing steps and centrifugation. At the end of this procedure, the cells from the petri dish were resuspended in 10 ml of culture medium. For further subculturing, 3 ml of the cell suspension was brought into a new petri dish, or 30 µl of cell suspension were used for the experiments. This number of cells would correspond to 70% of complete cell coverage of the sensors compared the situation in the petri dish at the beginning. Cell type dependent losses due to the preparation or subculturing process were not considered in this calculation.

At the beginning of an experiment, the IDES were brought into the measurement incubator (without CO_2) and filled with 120 µl of RPMI medium (10% FCS). After 1 h, the sensors were inoculated with 30 µl of cell suspension to monitor cell growth on the sensor.

At the end of any experiment, the IDES were trypsinised (enzymatic removal of cells) and cleaned with alcohol (70%) and ultrasonics for 10 min. After being rinsed with de-ionised water, they were dried for storage. Before another experiment, the IDES were disinfected with alcohol (70%) or autoclaved. They could be used several times without any restriction in function. So far, the sensors have been used up to 15 times and will be used further.

2.3 *Impedance measurements*

Impedance is the ratio of a sinusoidal voltage, applied across two terminals of a measurement cell, to the sinusoidal component of the current flowing between the terminals that results from the applied potential difference. Unless the system is purely resistive, impedance is a complex quantity, because the current has a different phase from the applied voltage:

$$\boldsymbol{Z} = Z' + iZ'' = |\boldsymbol{Z}|e^{i\Theta} \quad i = (-1)^{0.5} \tag{1}$$

If we consider a resistance R_{ser} and a capacitance C_{ser} in series, we find that the voltage is linked to the current through

$$\boldsymbol{Z} = R_{ser} - i/(\omega C_{ser}) \tag{2}$$

Sometimes it is advantageous to analyse AC circuits in terms of the admittance $\boldsymbol{Y}$, which is the inverse impedance, $1/\boldsymbol{Z}$, usually symbolised by

$$\boldsymbol{Y} = Y' + iY'' = |\boldsymbol{Y}|e^{-i\Theta} \tag{3}$$

This concept is especially useful in the analysis of parallel circuits, because the overall impedance of parallel elements, e.g. C_{par}, R_{par}, is simply the sum of the individual admittances

$$\boldsymbol{Y} = 1/R_{par} + i\omega C_{par} \tag{4}$$

All combinations of elements (Z', Z''), $(|Z|, \Theta)$, (C_{ser}, R_{ser}), (Y', Y''), $(|Y|, \Theta)$, (C_{par}, R_{par}) are equivalent and can be converted into each other.

The impedance measurements were carried out with a Stanford Research System SR 720 LCR-meter. The test frequencies were 100 Hz, 1 kHz, 10 kHz and 100 kHz. An oscillating potential of 100 mV was applied through a 100 kΩ resistor. The measurement current was therefore reduced to 1 µA or less. The current density was about 20 µA cm^2, if a uniform distribution of the current on the electrode area was assumed.

A computer-controlled eight-fold multiplex unit allowed examination of eight IDES in parallel. Therefore it was guaranteed that all sensors had undergone through the same treatment and were inoculated at the same time, with cells from the same cell culture plate. If different cell types were used, inoculation occurred at least at the same time. Effects of addition to the medium, exchange of medium and temperature could be compared. All measurements were computer controlled.

We selected an equivalent circuit with a conductance and a capacitance in parallel. The capacitance C_{par} at a frequency of 10 kHz was used to represent the measured data. The cell membrane still insulates at this frequency. However, other representations and frequencies also produce reasonable results (EHRET *et al.*, 1997), particularly if the requirements are changed, or the sensor geometry is optimised for other applications. The selected representation and frequency have nothing in common with any kind of modelling of the electrode–cell interface.

3 Results

Fig. 3 shows an experiment with 293, CV-1 and LS 174T cells that were cultured for 4 days on the IDES. Figs. 4 and 5 show the corresponding experiment with HeLa and RT112 cells. The cells were inoculated at the beginning of the experiment and started to attach, spread and divide on the electrodes. This could be seen as a decrease in C_{par}. At high C_{par} values, the signal-to-noise-ratio is rather high. At these impedance values, the fluctuations result from the LCR meter (with or without cells), which was proven with appropriate electrical circuits comprising a resistor and a capacitor (R_{par}, C_{par}). It is definitely not a biological effect, in contrast to fluctuations at lower C_{par} values.

The culture medium was completely changed in a cycle of approximately 24 h. Subsequently, the fresh medium triggered cell type specific reaction. After 12 h, CV-1 cells reached confluence, whereas the other cells needed 2 days until a complete monolayer was formed (optical observation). Nevertheless, the cells showed significant fluctuations even after 2 days. The fast changes in C_{par} after a change of the culture medium took approximately 15–20 min, as shown in Fig. 3*b*.

The addition of Triton X-100 was used at the end of all experiments for the determination of a so-called end-point. The very small difference between the end-point and a cell-free start-point was due to changes in the medium. They result

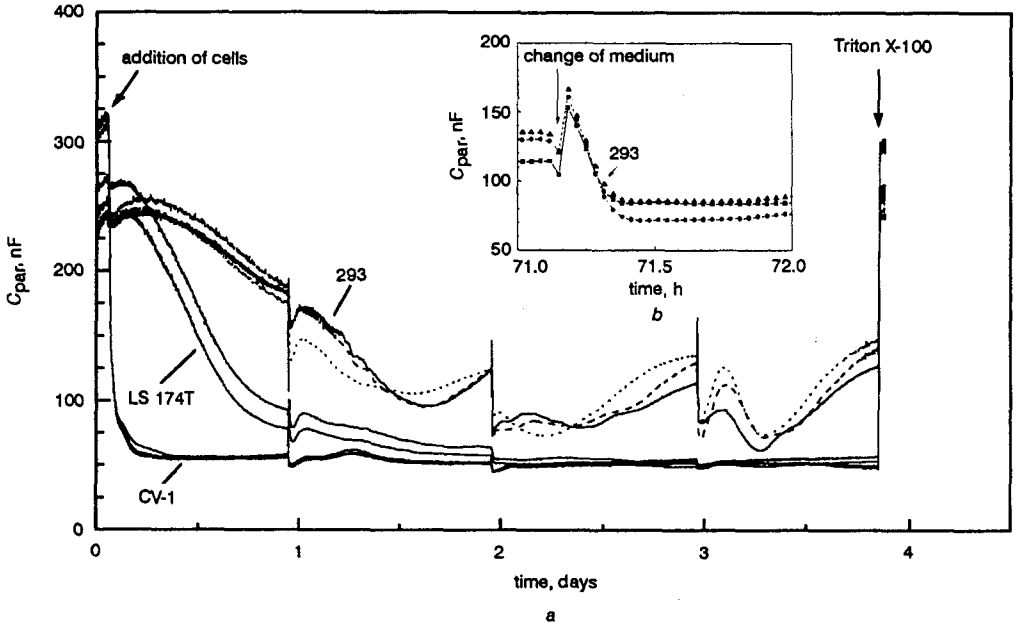

Fig. 3 *(a) Impedance measurement of eight IDES with 293 (3), LS 174T (2) and CV-1 (3) cells. Medium was changed daily and triggered cell type specific signal variations. (b) Enlargement showing change of medium after three days. Symbols have distance of 2 min.*

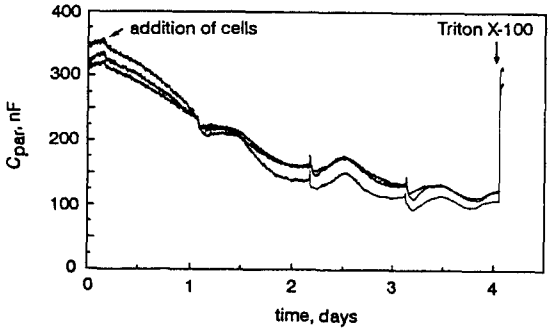

Fig. 4 *Impedance measurement of three IDES with HeLa cells. Medium was changed daily and triggered cell type specific signal variations. Measurements were performed together with experiment in Fig. 5, but were separated for clarity*

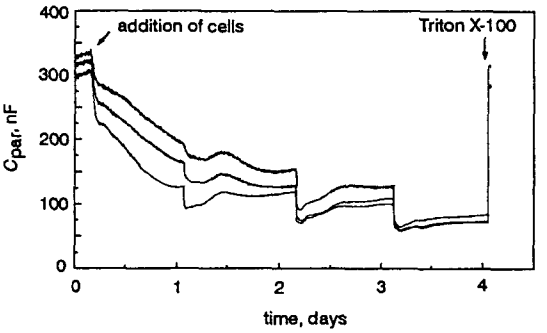

Fig. 5 *Impedance measurement of three IDES with RT112 cells. Medium was changed daily and triggered cell type specific signal variations. Measurements were performed together with experiment in Fig. 4, but were separated for clarity*

from effects of the cell metabolism and from the addition of substances (mainly Triton X-100).

4 Discussion

Cells adhere to other cells or to solid surfaces, despite being apparently separated by a gap of 10–20 nm (up to several 100 nm outside special contact zones). Improved staining methods in electron microscopy are capable of showing the glycocalyx. The glycocalyx consists of extracellular carbohydrate-linked segments of integral membrane glycoproteins. Direct molecular connections are established between the cell surface, presumably the glycocalyx, and the substrate. The contact zone can be viewed as an aqueous compartment spanned by the polycationic segments of membrane macromolecules and can act as a molecular sieve to the diffusion of aqueous molecules (GINGELL, 1990).

Biological cells are very poor conductors at low frequencies (at least below 10 kHz) and therefore force electrical currents to bypass them. If cells grow adherently on an electrode, this effectively reduces the electrode area, and the interface impedance is increased because it is inversely proportional to the total electrode area reached by the current (SCHWAN, 1963).

The total impedance of the system is very sensitive to the presence of and distance between the cells on the electrodes. This implies as well that most of the influences on the sensor signal have their origin in the cell layer that is closest to the electrodes. The influence of a second layer of cells, as in multilayer cultures, has not been examined up to now but should be low.

The influence of the cells on the impedance signal is clearly shown in Figs. 3–5. CV-1 cells show a very fast decrease in C_{par}, representing an increase in intact cell membranes in close contact with the electrodes. The signal does not change significantly for the rest of the experiment. As there have been only 70% of the cells necessary to form a complete monolayer, as in the petri dish, at least one division has to occur in the first hours of the experiment, or the amount of cells has been sufficient to form a monolayer with a lower cell density, and the divisions to reach the same density occur later on.

LS 174T cells showed no effects for the first hours. The signal variations were approximately the same as would be expected for a pure temperature effect in an experiment without cells (EHRET *et al.*, 1997). A significant decrease in C_{par} occurred at first after about 6 h. The change of medium after the first day resulted in a slight fluctuation in the sensor signal and a further decrease in C_{par}. After day two, there are no more significant fluctuations in the signal.

293 cells showed a very characteristic behaviour. The first adhesion was slow. After two days, a complete monolayer was formed, as could be seen by optical observation. The cells reacted with a considerable decrease in C_{par} after a change of medium. The inset in Fig. 3 shows that this decrease takes

about 15–20 min. After day three, there was a marked fluctuation in the sensor signal. At this time the cell layer was complete, and so these fluctuations could not only result from a pure increase in cell membranes near the electrodes but should have their origin in collective morphological changes in the adhesion of the cells to the substrate (or to other cells via tight junctions).

HeLa and RT112 cells also showed a typical behaviour. Both cell types formed a monolayer after two days. RT112 cells showed a fast first adhesion followed by a slower decrease in C_{par} and showed a strong decrease after changes of medium, whereas the effects on HeLa cells were not as pronounced, and the cells did not reach as low C_{par} values as the other cell lines. This may be due to a lower adhesion to the electrodes.

The fluctuations and/or reactions of the different cell lines to changes of the culture medium is of great interest. A very important component of the culture medium is serum. When cells are removed from their original tissue or organism and placed in culture, the medium must provide all the environmental conditions to which the cells have been exposed *in vivo*. Only then will they be able to survive, to proliferate and to differentiate. The extracellular medium must meet essential requirements for survival and growth (i.e. must provide nutritional, hormonal and stromal factors). Among the biological fluids that have proved successful for culturing cells outside the body, serum has gained the most widespread significance. Although the requirements that have led to the development of complex, chemically-defined media have been partially elucidated, 5–20% serum was, and still is, usually needed for optimum cell growth. For a variety of reasons it would be of great advantage to eliminate the indefinable serum constituents, thus creating fully chemically-defined media. For adherently growing cells, the intensity of adhesion is a very important test factor in this approach. Effects of serum on the cell culture could largely depend on the lot of serum used. Experiments concerning the influence of serum components in general have already been performed with IDES (EHRET *et al.*, 1997).

We have also tried to describe our results with equivalent circuits with components such as resistors, capacitors, Warburg impedances and constant phase elements using impedance spectroscopy (EHRET *et al.*, 1997). The effects of biological samples on interface impedances are very complex. A word of caution must be given whenever circuit models are developed from only the frequency dependence of measured impedance components. For any equivalent circuit consisting of three or more elements, another circuit can be found that consists of the same number of uniform elements but differs in the manner in which they are connected. This second circuit will have a frequency dependence identical to that of the first circuit over the entire frequency range (DEROSA and BEARD, 1977, MACDONALD, 1992). We therefore decided not to include a more detailed analysis of our data in terms of ideal electrical components such as ohmic resistors and parallel-plate capacitors. Perhaps some kind of mathematical modelling would be possible, as done by GIAEVER and KEESE (1993). Nevertheless, this problem should be studied further.

5 Conclusions

Impedance measurements on cellular systems with interdigitated electrode structures have been shown to be an effective way of monitoring cellular behaviour on-line and in real-time. The main effect of cells on IDES is due to the insulating property of the cell membrane. The presence of intact cell membranes on the electrodes and their distance from the electrodes determine the current flow and thus the sensor signal. Growth behaviour, adhesive properties and the physiological state of adherent cells can be monitored during cultivation. The cells show significant, cell type specific fluctuations in the impedance signal in the course of a day. As the sensor signal is produced by all cells on a sensor, it represents the collective behaviour of the cells.

IDES also have the capability to examine more complex reactions influencing shape, morphology and the surroundings (e.g. the extracellular matrix) of the cells. Such influences can result from different sera, growth factors, hormones, antibodies, cytostatics or other chemical/physical/biological manipulations.

IDES can also provide useful information about culture conditions for biotechnological production of adherent cells (e.g. skin culture for transplantation purposes). Adherently growing cellular systems cultured on IDES also have an application as cell-based biosensors. However, a more complete description of cellular behaviour is only possible if different sensor types are used.

The PhysioControl-Microsystem (PCM) provides sensors for pH and O_2 and permits high-resolution light microscopy. In this context, IDES are an important sensoric component to complete the information provided by the PCM (WOLF *et al.*, 1998). IDES can control the adherence of cells. They can be applied for the control of stably transfected cells (product formation), if adherence has been an important feature of the non-transfected cells and this feature should be maintained for the transfected cells.

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Authors' biographies

Ralf Ehret received his Diploma in Physics (electron spectroscopy with synchrotron radiation) in 1991, and his PhD in Biology, in 1997. Since 1992 he has been a member of Professor Wolf's group. His scientific interests include the detection of cellular behaviour with microsensor, optical and scanning electron microscopy techniques. His special interests are the adhesive features of cells and related phenomena for exploitation in microsensor systems.

After a three year apprenticeship in electromechanics and electronics, Werner H. Baumann worked for two years in a research laboratory for magnetic materials for Robert Bosch GmbH, Buehl, Germany. Afterwards he studied Physics in Freiburg and received a Diploma in 1991, and graduated in Biology in 1996. Since 1992 he has worked at the Institute of Immunobiology in Freiburg. His main scientific interests are the development of silicon sensor chips, sensor electronics and fluid handling systems for measurement on living cells with microsensor systems (especially with ISFETs and CPFETs).

Martin Brischwein received his Diploma in Biology in 1992, from the University of Freiburg. Afterwards he joined Professor Wolf's group at the Institute for Immunobiology. He is working on the detection of cellular metabolism by various microsensor techniques. Since 1996, he has been employee of PTS Ltd, Freiburg, which is involved in the development and marketing of microsensor systems for *in vitro* monitoring of living cells.

Anne Schwinde, a Technical Assistant, has worked in several departments of the University of Freiburg since 1970, including the limnology, cell biology, pathology and immunobiology departments. Her special interests include cell culture and electron microscopy techniques.

Bernhard Wolf is Professor for Medical Physics at the University of Freiburg. His main interests include analytical electron microscopy techniques (e.g. EELS) and the development of a microsensor-based system for on-line recording of cellular behaviour in the field of cancer research and related areas.