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# Development of ISFET array-based microsystems for bioelectrochemical measurements of cell populations

Sergio Martinoia <sup>a,\*</sup>, Nicola Rosso <sup>a</sup>, Massimo Grattarola <sup>a</sup>, Leandro Lorenzelli <sup>b</sup>, Benno Margesin <sup>b</sup>, Mario Zen <sup>b</sup>

<sup>a</sup> Department of Biophysical and Electronic Engineering (DIBE), University of Genova, Via all'Opera Pia 11A, 16145 Genova, Italy <sup>b</sup> ITC-irst Microsystems Division, Via Sommarive 18, Povo 38050, Trento, Italy

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#### Abstract

Monitoring the bioelectrochemical activity of living cells with sensor array-based microsystems represents an emerging technique in a large area of biomedical applications, ranging from basic research to various fields of pharmacological analyses. The main appeal is the ability of these miniaturised microsystems to perform, in real time, non-invasive in-vitro investigations of the physiological state of a cell population. In this paper, we present two different microsystems designed for multisite monitoring of the physiological state of a cell population. The first microsystem, intended for cellular metabolism monitoring, consists of an array of 12 spatially distributed ISFETs to detect small pH variations induced by the cell population. The second microsystem consists of an array of 40 ISFETs and 20 gold microelectrodes and it has been designed to monitor the electrical activity of neurons. This is achieved by direct coupling of the neuronal culture with the ISFET sensitive layer and by utilising gold microelectrodes for neuronal electrical stimulation. © 2001 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

In the last decade, metallic microelectrode arrays for electrophysiological activity recording from neurons in culture have been designed and optimised (Gross et al., 1985; Jimbo et al., 1993; Martinoia et al., 1993; Wilson et al., 1994; Meister et al., 1994). Arrays of ISFETs have also been recently proposed for similar applications (Offenhausser et al., 1997).

Moreover, advances in short term in-vitro culturing of neurons have offered to the neuroscientific community the opportunity to test, in a controlled environment, fundamental hypotheses concerning nerve regeneration and the formation of specific patterns of connection during the development of a network of neurons (Nelson et al., 1989; Bulloch and Syed, 1992; Hockberger et al., 1993). Recently, the combination of micromachining technology, new microtransducer realisation methodologies and the aforementioned in-vitro culture techniques, has allowed the study of the behaviour of neuron cultures grown on an appropriately functionalised substrate in a controlled and pre-conditioned way (Curtis et al., 1992; Denyer et al., 1997; Maher et al., 1999). Thus, real time in-vitro investigations of the physiological state of a cell population in a broad area of biomedical applications, ranging from basic research to various fields of pharmacological analyses, can be performed.

Complex systems, constituted by arrays of chemical and electrophysiological transducers also combining micromachined structures for cell culture guidance, can be envisaged. The design of such advanced microsystems demands the availability of fabrication processes able to integrate different technologies, for example those utilised for the fabrication of ISFET chemical sensors (Harame et al., 1987; Martinoia et al., 1999), metallic microelectrodes and micromachined three-dimensional structures.

In this paper, we present two different microsystems designed for multisite monitoring of the physiological

<sup>\*</sup> Corresponding author. Tel.: + 39-010-353-2251; fax: + 39-010-353-2133.

E-mail address: martinoia@dibe.unige.it (S. Martinoia).

state of a cell population. The first microsystem, intended for cellular metabolism monitoring, consists of an array of 12 ISFETs spatially distributed to detect small pH variations induced by the cell population, two temperature sensors and an interdigitated two-electrode structure. The second microsystem consists of an array of 40 ISFETs and 20 gold microelectrodes and has been designed to monitor the electrical activity of neurons. This is achieved by direct coupling of the neuronal culture with the ISFET sensitive layer and by utilising the gold microelectrodes for the electrical stimulation of the neurons.

The following paragraphs will present the developed technology and the two proposed microsystems together with possible applications and simulation results. The design methodologies and the results obtained through simulations will be then shown in detail.

# 2. Fabrication process

The design of chemical sensor based microsystems demands a fabrication process both technologically reliable and economically suitable to be integrated with different technologies, such as microelectrode fabrication and micromachining.

The technological process developed and reported here, consisted of a modified ion sensitive field effect transistor/complementary metal nitride oxide semiconductor (ISFET/CMNOS) technology based on a 4- $\mu$ m Al gate p-well CMNOS process. One of the main features was the use of a Si<sub>3</sub>N<sub>4</sub>/SiO<sub>2</sub> double layer as a gate dielectric for fabricating both the ISFET sensors and all the integrated MOSFET devices. The chemical sensing layer was made of stoichiometric Si<sub>3</sub>N<sub>4</sub> deposited at 775 °C in a LPCVD system (ASM DFR210) using ammonia and dichlorosilane in a 4:1 flow ratio. In the current release of the fabrication process, two new mask levels were implemented to produce the microelectrodes and the passivation polymeric layers. The microelectrodes were produced by deposition of 200 nm of gold (by evaporation) onto a 10 nm chromium layer. The function of the chromium layer was to promote gold adhesion and the gold electrodes were defined by wet etching. In the final step of the fabrication process, a photopolymeric passivation layer (SU8) was spun onto the whole wafer surface exposed and then developed. Fig. 1 shows the cross-section of an ISFET–gold microelectrode combined structure realised using the above mentioned technology.

# 2.1. ISFET sensor and gold microelectrode characteristics

The ISFET sensors are well-established Si<sub>3</sub>N<sub>4</sub>-gate ISFETs (Cambiaso et al., 1996; Martinoia et al., 1999) with a typical sensitivity of 56 mV/pH, a drift of 600  $\mu$ V/h and a temperature dependence of  $\approx 1.5$  mV/K at constant pH. The ISFET channel width to length ratio (W/L) was 50 for all the sensors intended for the cellular metabolism monitoring applications, whereas the ISFETs in the sensor chip to monitor the electrical activity of neurons were designed with spatially diversified geometrical W/L factors (W/L = 2, 5, 10, 20). Fig. 2(a) shows an enlarged microphotography of a  $40 \times 40$  $\mu$ m gold microelectrode, where the 36  $\times$  36  $\mu$ m opening in the SU8 passivation layer has been magnified. Fig. 2(b) shows the two-electrode interdigitated structure (40 fingers with length = 1 mm, width = 20  $\mu$ m and electrode spacing = 20 um) produced to perform impedance measurements (e.g. to evaluate cellular adhesion). Both the planar microelectrode array and the interdigitated electrodes were implemented within the ISFET array sensor chip.

#### 3. Microsystem for cellular metabolism monitoring

A completely automated microsystem was designed and fabricated for cellular metabolism monitoring. It



Fig. 1. Cross-section of the ISFET sensor with the gold microelectrode layer.



Fig. 2. Planar gold structures: (a)  $40 \times 40 \ \mu m$  gold microelectrode, (b) two-electrode interdigitated gold structure.

was controlled by a personal computer via a generalpurpose acquisition board (National Instruments AT-MIO 16E). Software tools (LabWindows/CVI-National Instruments) were developed to allow, during the validation phase, easy configuration and fine-tuning of the control parameters.

The system prototype consists of a sensor chip, encapsulated by a SMD package on a printed circuit board positioned inside a flow injection microchamber. An electronic front end controls the flow on/off phases, the injected analyte selection, the sensor signal conditioning and the sensor multiplexing. Cell cultures can be grown either, by traditional laboratory methods, on the membrane of commercial wells (Transwell<sup>®</sup> Cell Culture Permeable Supports) positioned onto the sensor chip surface or directly onto the chip sensor surface. Moreover, non-adhering cell populations can be entrapped, by slightly modifying the chamber structure, between two culture permeable membranes.

Fig. 3 shows a complete technical drawing of the head stage (the core) of the flow injection microcham-

ber and sensor chip assembly. Fig. 4 shows, as an example, the software user interface with a running measurement for three sensors.

#### 3.1. Sensor chip

The sensor chip to monitor the metabolic activity of cellular populations was a  $1.75 \times 1.85$  cm die with 12 ISFETs, two temperature sensors and a conductivity sensor. The chip was also provided with a metallic shield to prevent ESD effects. The ISFETs monitor, by means of a measure of pH variation, the medium acidification induced from the cellular metabolic activity; the temperature sensors verify the medium temperature imposed by an external thermostatic system. The additional conductivity sensor, an interdigitated two-electrode structure, can monitor cell culture adhesion (Giaver and Keese, 1993), allowing the microsystem to be used for experimental applications where a cell population is directly grown onto the chip surface.

The cell culture adhesion can be monitored by measuring the impedance changes across an interdigitated electrode structure as shown in the literature (Ehret et al., 1998). These changes can be easily correlated with the detachment of the adherent culture and therefore with a decrease of the cell metabolic activity. Fig. 5 shows the layout of the fabricated ISFET sensor array.

#### 3.2. Microsystem simulation results

The ISFET sensors were designed according to the results of preliminary SPICE simulations performed in



Fig. 3. Flow injection microchamber head stage and sensor chip package.



Fig. 4. Front panel of the software user interface.

static and transient analysis mode utilising an ISFET macromodel previously developed (Martinoia and Massobrio, 2000). The design of ISFET sensors with large drain/source regions demanded a first set of SPICE simulations entirely devoted to observe the effect of different drain/source resistance values on the sensor working point.

Once defined the electrical sensor operating conditions, a simplified multiplexing configuration (shown in Fig. 6) was adopted to simulate the ISFET switching behaviour for different pH values. The sensors were connected, in follower mode, to a common high compliance current sink ( $I_{sink} = 110 \ \mu A$ ) and to a NMOS output stage. In the simulated circuit three nMOS devices (M1, M2 and M3 in Fig. 6) were also employed as switches. Then, in a second set of SPICE simulations, the whole circuit was simulated in transient analysis mode to have some indications about the sensor signal stability. SPICE simulation results, for the multiplexing configuration, are given in Fig. 7. The simulation results show an almost linear range of variation of the output voltage  $(V_{out})$  for ISFETs experiencing different pH values and a good response stability for the drain current polarisation used ( $I_{sink} = 110 \ \mu A$ ).

The simplified equation used to evaluate the pH variation depending on the metabolic activity is (Bousse et al., 1992; Owicki and Parce, 1992):

$$\frac{\mathrm{d}\mathbf{p}\mathbf{H}}{\mathrm{d}t} \cong \frac{C_{\mathrm{den}} \cdot [\mathbf{H}^+]}{D \cdot \beta}$$

 $\frac{dpH}{dt} \cong \frac{C_{den} \cdot [H^+]}{D \cdot \beta}$  where  $C_{den}$  is the number of cells per unit area,  $[H^+]$  is the moles/second proton generation rate, D is the cell population-sensor distance and  $\beta$  is the buffer capacity of the medium. Typical values are  $C_{den} = 2 \times 10^5$  cell/cm<sup>2</sup>,  $H^+ = 10^8$  protons/s (for mammalian fibroblasts (Owicki and Parce, 1992)), D = 200µm and  $\beta = 5 \times 10^{-3}$  moles/l, which corresponds to a  $\Delta pH \cong 0.05$  per minute. With these figures, a variation of 2.5 mV/min is obtained as output voltage of the sensor indicating therefore that cell metabolism rate can be easily monitored on this time scale (i.e. minutes).

The microchamber prototype, towered above the sensor chip, was designed with a central inlet and lateral fluid outlets. This geometry was expressly studied to ensure a radially distributed flow with low turbulence effects onto the sensor area. The microchamber design hypotheses were formulated neglecting the structural deformation of the membrane support. The fluidic performances were tested with the ANSYS/FLOTRAN finite element analysis simulator. The calculus steps were optimised designing, in the ANSYS user interface, the microchamber transversal section and employing a rotational geometry based procedure to reduce the simulation problem to a two-dimensional case. For the



Fig. 5. Layout of the ISFET array sensor chip for metabolic activity monitoring. Detail of the ISFET gate area.

flow profile study, the applied boundary conditions were the inlet initial fluid velocity and the zero pressure value at the outlets. The graph in Fig. 8 shows the simulation results for a nodal analysis performed in laminar fluid flow conditions, the magnitude of the velocity vectors is represented with different colours. In the simulations, a low velocity vector magnitude gave us the indications in order to arrange the ISFET sensors onto the sensor chip area without turbulence: the distance of 4 mm between sensor area and inlet was considered a good compromise for the estimated fluid rates.

#### 4. Microsystem for neural electrical activity monitoring

An automated system based on new (i.e. mixed) microtransducer arrays consisting of conventional gold microelectrodes and ISFET microsensors has been designed and realised.

The system is intended for electrophysiological measurements by monitoring, extracellularly, the electrical activity from a network of neurons coupled to the microtransducer array substrate. It has been shown that electrophysiological activity from cells can be measured by a FET based sensor (Fromherz et al., 1991). The proposed system should be able to measure the electrical activity, as well as the metabolic activity, of a neuronal cell population by means of ISFET devices. Moreover, the electrical activity of the neurons can be stimulated (and also recorded) by using the conventional gold microelectrodes.

# 4.1. Sensor chip

The sensor chip, fully devoted to perform electrophysiological measurements on neuron populations, was a  $2.2 \times 3.7$  cm die with two merged arrays of  $8 \times 5$ 



Fig. 6. ISFET array multiplexing circuit.



Fig. 7. SPICE simulations. Multiplexing circuit transient analysis results for pH = 4, 7, 10 and Io = 100  $\mu$ A.

ISFET sensors and  $4 \times 5$  gold electrodes. The chip dimensions were chosen to ensure a large area for cell culturing and to make the device easy to handle.

Fig. 9 shows the sensor chip. The large area  $(1.5 \times 1.5 \text{ mm})$  electrode connecting pads have been designed to enable the bare sensor chip to be utilised with a custom connecting/switching box apparatus.

#### 4.2. Microsystem simulation results

Computer simulations were performed with respect to variation of parameters involved in electrical transduction between neuron and ISFET. The neuron-transistor junction was modelled following circuit models previously developed (Grattarola and Martinoia, 1993). Fig. 10 shows the simulation results of extracellularly recorded signals from a neuron for different values of the coupling impedance  $(R_{seal})$  between the neuronal membrane and the transducer (Bove et al., 1996) and for different values of the insulator layer thickness. The simulation results prove the possibility of recording signals up to a few microamperes for the considered insulator thickness. Those data were used to choose the insulator thickness values (Ox: silicon dioxide = 30 nm; Nit: silicon nitride = 100 nm) for the fabricated ISFET sensors.

#### 5. Conclusions and prospects

Two microsystems have been developed devoted to the bioelectrochemical characterisation of a cell population in culture. The two proposed systems allow shortand long-term in-vitro physiological studies to be performed under controlled conditions. The main appeal of the proposed techniques and methodologies is the possibility of a stable coupling between a functionalised substrate and a cell population. The proposed microsensor arrays work as a kind of new Petri's dish which permits one to follow, in a controlled condition, both the metabolic activity of a cell culture and, for



Fig. 8. ANSYS/FLOTRAN microchamber fluidic simulation results: nodal analysis in laminar fluid flow condition. The magnitude of the velocity vectors is represented with different colours.



Fig. 9. ISFET array sensor chip for electrophysiological measurement on neuron populations. Detail of the  $5 \times 5$  mm active area sensor chip and of the ISFET-electrode pair.

excitable cells, the metabolic and the electrophysiological activities. In addition, the temperature of the culture environment and the cell culture adhesion can be monitored by means of appropriate sensors. The proposed microsystems have been designed taking advantage of specialised simulation tools both for the microfluidics and the microsensor arrays. The main parameters of the fabrication have been adjusted following the indications obtained from the simulation results.

Future work will be devoted to the validation of the proposed automated microsystem with particular emphasis on pharmacological applications and to the integration of cell guidance methodologies to re-designed micromachined microsystems.



Fig. 10. Simulation results of extracellularly recorded signals from a neuron for two values of the coupling impedance: (a)  $R_{\text{seal}} = 100 \text{ M}\Omega$  and (b)  $R_{\text{seal}} = 1 \text{ G}\Omega$ .

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#### References

- Bove, M., Martinoia, S., Grattarola, M., Ricci, D., 1996. The neuronmicrotransducer junction: linking equivalent circuit models to microscopic description. Thin Sol. Films 284–285, 772–775.
- Bousse, L., Owicki, J.C., Parce, J.W., 1992. Biosensors with microvolume reaction chambers. In: Yamamauchi, S. (Ed.), Chemical Sensors Technology, vol. 4. Elsevier, Amsterdam, pp. 145–166.
- Bulloch, A.G.M., Syed, N.I., 1992. Reconstruction of neuronal networks in cultures. Trends Neurosci. 15, 422–427.
- Cambiaso, A., Chiarugi, S., Grattarola, M., Lorenzelli, L., Lui, A., Margesin, B., Martinoia, S., Zanini, V., Zen, M., 1996. An H+-FET-based system for on-line detection of microorganism in waters. Sens. Actuat. B34, 245–251.
- Curtis, A.S.G., Breckenridge, L., Connolly, P., Dow, J.A.T., Wilkinson, C.D.W., Wilson, R., 1992. Making real nets: design and criteria. Med. Biol. Eng. Comput. 30, CE33–CE36.
- Denyer, M.C., Britland, S.T., Curtis, A.S.G., Wilkinson, C.D.W., 1997. Patterning living neural networks on microfabricated electrophysiological recording devices. J. Cell. Eng. 2, 122–130.
- Ehret, R., Baumann, W., Brischwein, M., Schwinde, A., Wolf, B., 1998. On-line control of cellular adhesion with impedance measurements using interdigitated electrode structures. Med. Biol. Eng. Comput. 36, 365–370.
- Fromherz, P., Offenhausser, A., Vetter, T., Weis, J.A., 1991. Neuronsilicon junction: a Retzius cell of the leech on an insulated-gate field-effect transistor. Science 252, 1290–1293.
- Giaver, I., Keese, C.R., 1993. A morphological biosensor for mammalian cells. Nature 336, 591–592.
- Grattarola, M., Martinoia, S., 1993. Modeling the neuron-microtransducer junction: from extracellular to patch recording. IEEE Trans. Biomed. Eng. 40, 35–41.
- Gross, G.W., Wen, W., Lin, J., 1985. Transparent indium-tin oxide patterns for extracellular, multisite recording in neuronal cultures. J. Neurosci. Methods 15, 243–252.

- Harame, D.L., Bousse, L., Shott, D.L., Meindl, J.D., 1987. Ion sensing devices with silicon nitride and borosilicate glass insulator. IEEE Trans. Elect. Dev. 34, 1700–1707.
- Hockberger, P.E., Racker, D.K., Houk, J.C., 1993. Culturing neural networks. In: Stenger, D.A., McKenna, T.M. (Eds.), Enabling Technologies for Culturing Neural Networks. Academic Press, New York, pp. 35–49.
- Jimbo, Y., Hugh, P., Robinson, C., Kawana, A., 1993. Simultaneous measurements of intracellular calcium and electrical activity from patterned neural networks in culture. IEEE Trans. Biomed. Eng. 40 (8), 804–810.
- Maher, M.P., Pine, J., Wright, J., Tai, Y.C., 1999. The neurochip: a new multielectrode device for stimulating and recording from cultured neurons. J. Neurosci. Methods 97, 45–56.
- Martinoia, S, Massobrio, G., 2000. A behavioral macromodel of the ISFET in SPICE. Sens. Actuat. B 62, 182–189.
- Martinoia, S, Bove, M., Carlini, G., Ciccarelli, C., Grattarola, M., Storment, C., Kovacs, G.T., 1993. A general purpose system for long-term recording from a microelectrode array coupled to excitable cells. J. Neurosci. Methods 48, 115–121.
- Martinoia, S., Lorenzelli, L., Massobrio, G., Margesin, B., Lui, A., 1999. A CAD system for developing chemical sensor-based microsystems with an ISFET-CMOS compatible technology. Sens. Mater. 11–5, 279–295.
- Meister, M., Pine, J., Baylor, D.A., 1994. Multi-neuronal signals from the retina: acquisition and analysis. J. Neurosci. Methods 51, 95–106.
- Nelson, P., Yu, C., Fields, R.D., Neale, E., 1989. Synaptic connections in vitro: modulation of number and efficacy by electrical activity. Science 244, 585–587.
- Offenhausser, A., Sprossler, C., Matsuzawa, M., Knoll, W., 1997. Field-effect transistor array for monitoring electrical activity from mammalian neurons in culture. Biosens. Bioelectron. 12 (8), 819– 826.
- Owicki, J.C., Parce, J.W., 1992. Biosensors based on the energy metabolism of living cells: the physical chemistry and cell biology of extracellular acidification. Biosens. Bioelectron. 7, 255–272.
- Wilson, R.J.A., Breckeridge, L., Blackshaw, S.E., Connolly, P., Dow, J.A.T., Curtis, A.S.G., Wilkinson, C.D.W., 1994. Simultaneous multisite recordings and stimulation of single isolated leech neurons using planar extracellular electrode arrays. Biosens. Bioelectron. 53, 101–110.