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Microfabrication and characterization of an ion-selective microelectrode array platform

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Abstract

The design and the fabrication of an ion-selective electrode array platform aimed at in vitro intracellular recording are presented. The platform is composed of two parts: (i) a glass chip with microchannels whose bottom part is patterned with a platinum layer and (ii) a silicon chip in which an array of 50 μm long silicon nitride micropipettes with an inner diameter of 5 μm are structured. The 24 micropipettes are individually filled with a Ca^{2+} selective membrane, based on the neutral ionophore ETH 129. Preliminary tests in Ca^{2+} buffered solutions have shown a linear range from 10^{-6} to 0.1 M Ca^{2+} with a sensitivity of 27 mV/decade.

1. Introduction

Since the 1980s, the measurement of ion activities in living tissues is subject of an intense research effort. In particular the intracellular calcium activity is highly regulated: rapid oscillatory local changes in calcium concentration control numerous cellular enzymes and propagation of these changes as intracellular calcium waves functions as a signaling messenger [1]. Detection and measurement of intracellular ion concentrations have relied on various methods. Historically, Ca^{2+}-selective electrodes have been used concomitantly with absorption indicators [2]. However, interest in these indicators and in ion-selective electrodes (ISEs) has been eclipsed by the introduction, in the mid-eighties, of fluorescent Ca^{2+} probes such as indo-1, fura-2, and fluo-3 [2,3]. Despite the incontestable benefits of fluorescence imaging, ion-selective electrodes present some unique advantages, such as their almost unaltered behavior by the intracellular environment, their possibility to determine a wide concentration range of Ca^{2+} and the unchanged actual intracellular Ca^{2+} concentration [2,4–6].

Standard glass capillary ion-selective electrodes have been extensively used for both extra and intracellular measurements [4,5]. In order to gain better understanding of the cell physiology on the network level it would be a great advantage to be able to perform parallel measurements of a number of cells in the culture. Although the glass capillaries can be made very small, this technology is not very convenient for the realization of arrays and therefore, the measurements are limited to a few cells only. A number of research groups and several companies are investigating parallel patch-clamp recordings on planar surfaces [7–10]. Arrays of ion-selective electrodes have also been proposed for the detection of K^+ and Ca^{2+} in biological samples [11]. However, these electrodes, with an active area of 0.03 mm^2, are not suitable for intracellular measurements. In Ref. [12] a microfabricated sensor array is described that is sensitive towards pH and K^+. This Kapton-based array is used for in vivo measurements in a beating heart. The smallest single sensors had internal reference elements with a diameter of 0.25 mm.

Our efforts have been aimed at developing arrays of micropipettes, with tip diameters corresponding to the conventional glass ion-selective electrodes, that is about 0.5–2 μm. The final aim of our work is to use these arrays of ion-selective microelectrodes for the intracellular measurements of Ca^{2+} activities in hepatocyte cell cultures. In order to evaluate the technological feasibility, the design and the microfabrication of arrays of slightly larger ion-selective microelectrodes (5 μm inner diameter) has been carried out. Micropipette technology for such dimensions was investigated by a few research groups. Fujita and

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coworkers [13] developed a technology to create silicon oxide micropipettes for injection of genetic materials into cells, whereas Luginbuhl et al. [14] fabricated silicon oxide micro nozzles dedicated to the formation and injection of femtoliter size droplets for DNA mass spectrometry.

The approach described in this paper is similar to that presented in Ref. [13], except that the micropipettes are made of low stress silicon nitride which is stiffer than thermal oxide and thus, allows the fabrication of thinner micropipette walls. In this paper, we report on the fabrication of array of silicon nitride micropipettes realized by a combination of KOH and DRIE etchings as well as a directional SF_6 reactive ion etching. Furthermore, preliminary tests regarding the calcium sensitivity were performed.

2. Principle, design features, and fabrication

2.1. Principle and design of the microsystem

The schematic view of the ion-selective microelectrode array platform is depicted in Fig. 1. It is composed by three parts: a reservoir, containing the physiological solution medium and the cell culture, a silicon part in which the micropipettes are structured, and a Pyrex bottom plate that contains the microchannels with Pt microelectrodes. The ion-selective membrane cocktail is drawn in the microchannels by capillary forces. The Pt microelectrode is the internal contact of the ISE. Although this interface is not well defined, Pt has been chosen to simplify the technology. For

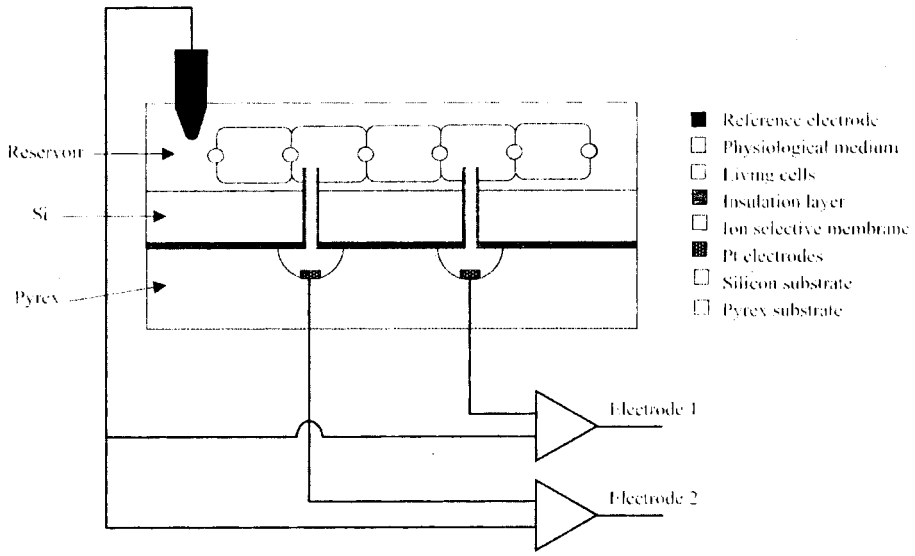

Fig. 1. Schematic cross-section of the platform for in vitro intracellular recording of ion concentration, designed for hepatocyte cell culture.

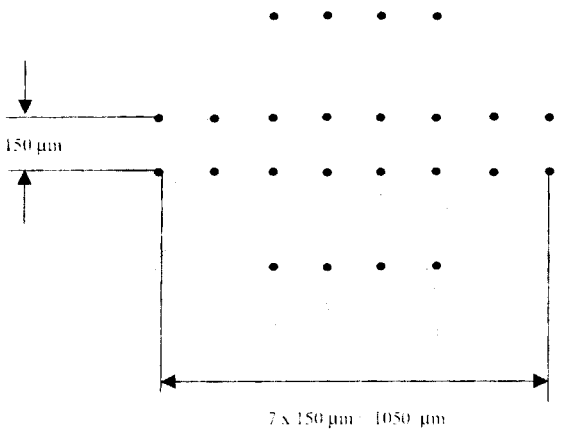

Fig. 2. Schematic design of the array of microelectrodes, and of the microchannels that allow the distribution of the ion-selective membrane into the micropipettes.

the next generation, a better defined interface, for example Ag/AgCl, will be used. The measurements are performed using a conventional Ag/AgCl reference electrode. The design consists of 24 micropipettes arranged in a 2 × 8 array with 150 μm spacing and four additional electrodes located on both sides of the array at 300 μm (Fig. 2). The micropipettes are designed with an inner diameter of 5 μm, and have a total length of 50 μm, of which a few microns protrude from the silicon surface.

2.2. Fabrication

Fig. 3 illustrates the fabrication steps of the micropipettes in a (1 0 0) silicon substrate (Fig. 3, steps i–v), as well as the fabrication of the microchannels with the metallic electrodes in a Pyrex substrate (steps vii–viii). The design consists of an array of 24 cylindrical cavities (50 μm deep with a diameter of 5 μm) made by DRIE in silicon (step i). After DRIE, a 500-nm thick low stress Si_xN_y layer is deposited by a LPCVD process (deposition temperature: 800 °C) (step ii). Then, the silicon is thinned down by KOH etching (40%, 60 °C) on the top side to provide a protection cavity for the micropipettes (step iii). The remaining silicon nitride is removed by SF_6 plasma etching to allow a subsequent thinning of the top side silicon until the nitride tips are exposed (step iv). The nitride caps of the tips are then etched via reactive ion etching and finally, a thin thermal oxide (200 nm) layer is grown onto the top side silicon surface (step v).

The second part of the platform is made of a Pyrex wafer in which 20 μm deep microchannels are HF-etched (20% HF), by using a 400-nm thick polysilicon mask (step viii). A 150-nm thick Ti/Pt layer is deposited by e-gun evaporation and then patterned at the bottom of the microchannels by a lift-off technique (step vii). The photoresist was spray coated

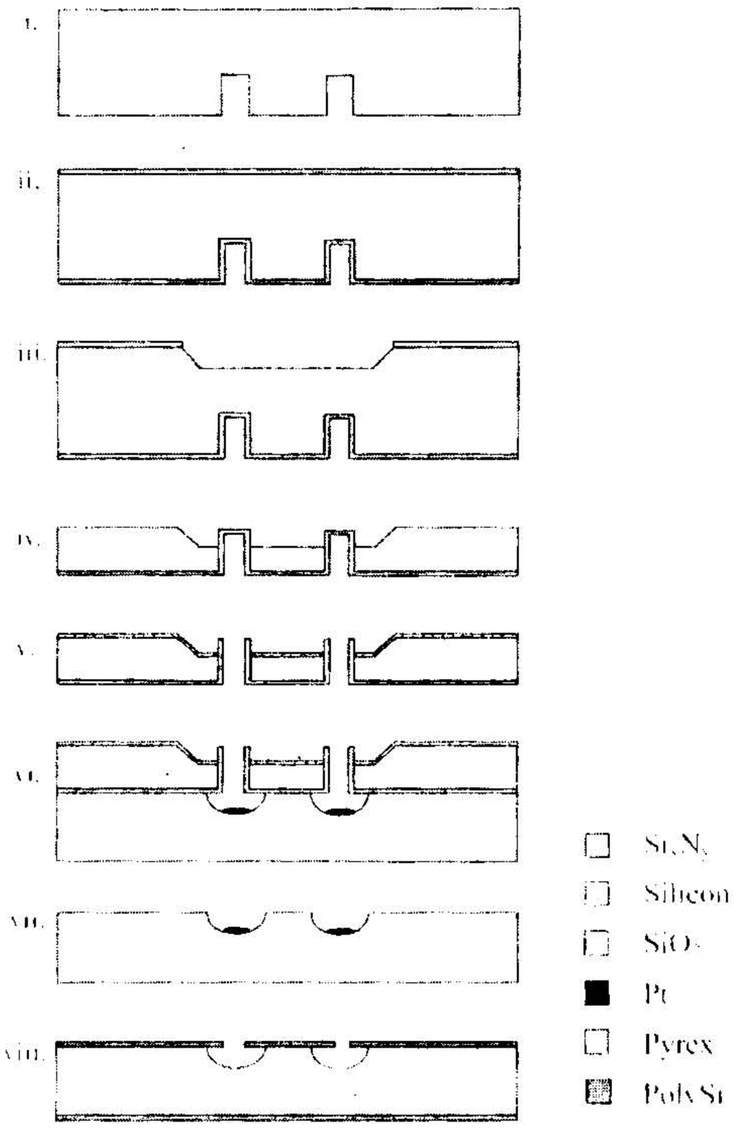

Fig. 3. Overview of the fabrication steps of the platform for ion-selective microelectrodes array. The micropipettes are etched in silicon by DRIE, coated with a silicon nitride layer and bonded onto a Pyrex plate, with HF-etched microchannels (see text for details).

to insure a homogeneous layer on the whole wafer, especially in the microchannels. Finally, the 50-μm thick silicon chip is anodically bonded (1000 V at 450 °C during 2 min) on the Pyrex substrate (step vi).

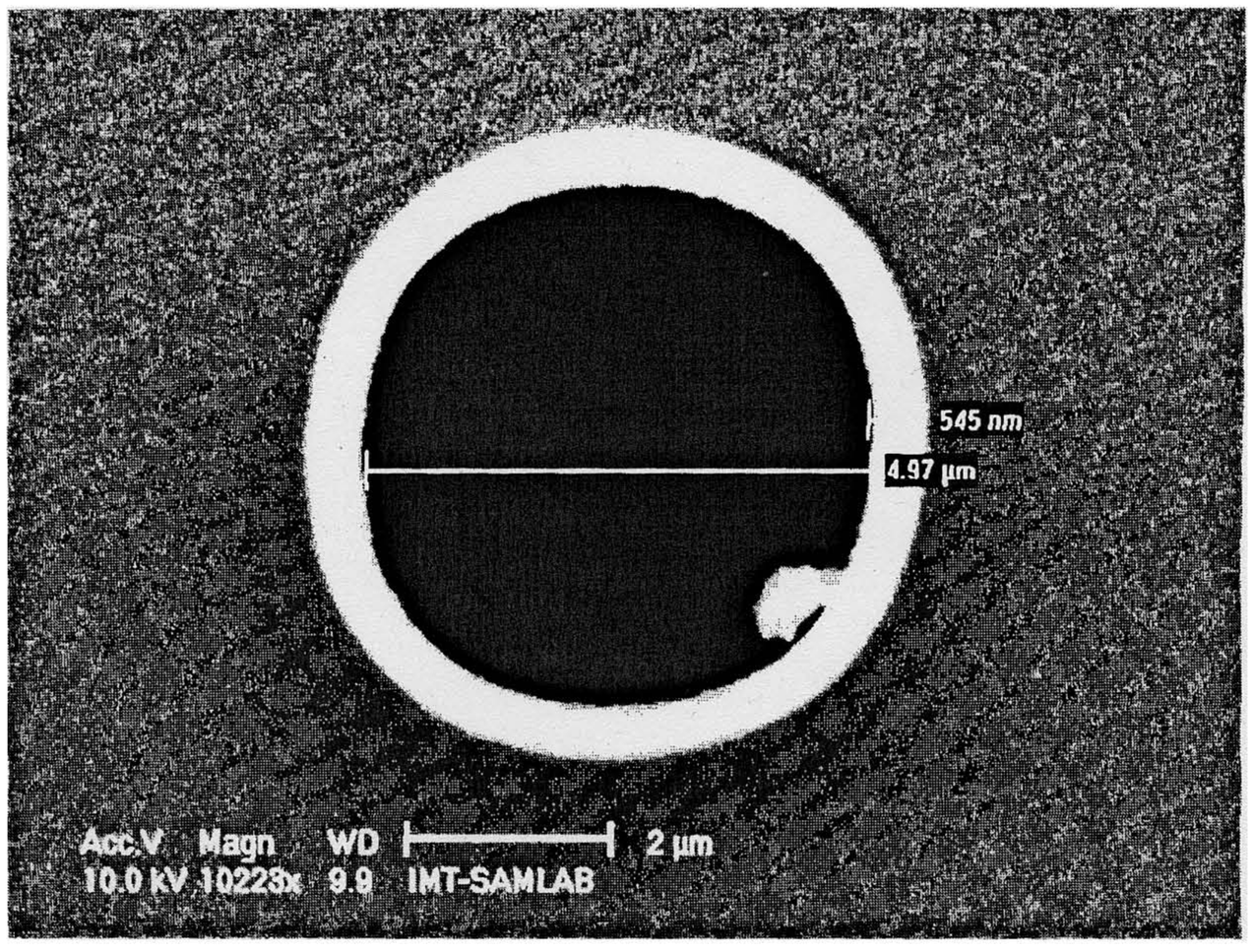

Fig. 4. Top view (SEM picture) of a Si_3N_4 micropipette, with 5 μm inner diameter and 6 μm outer diameter.

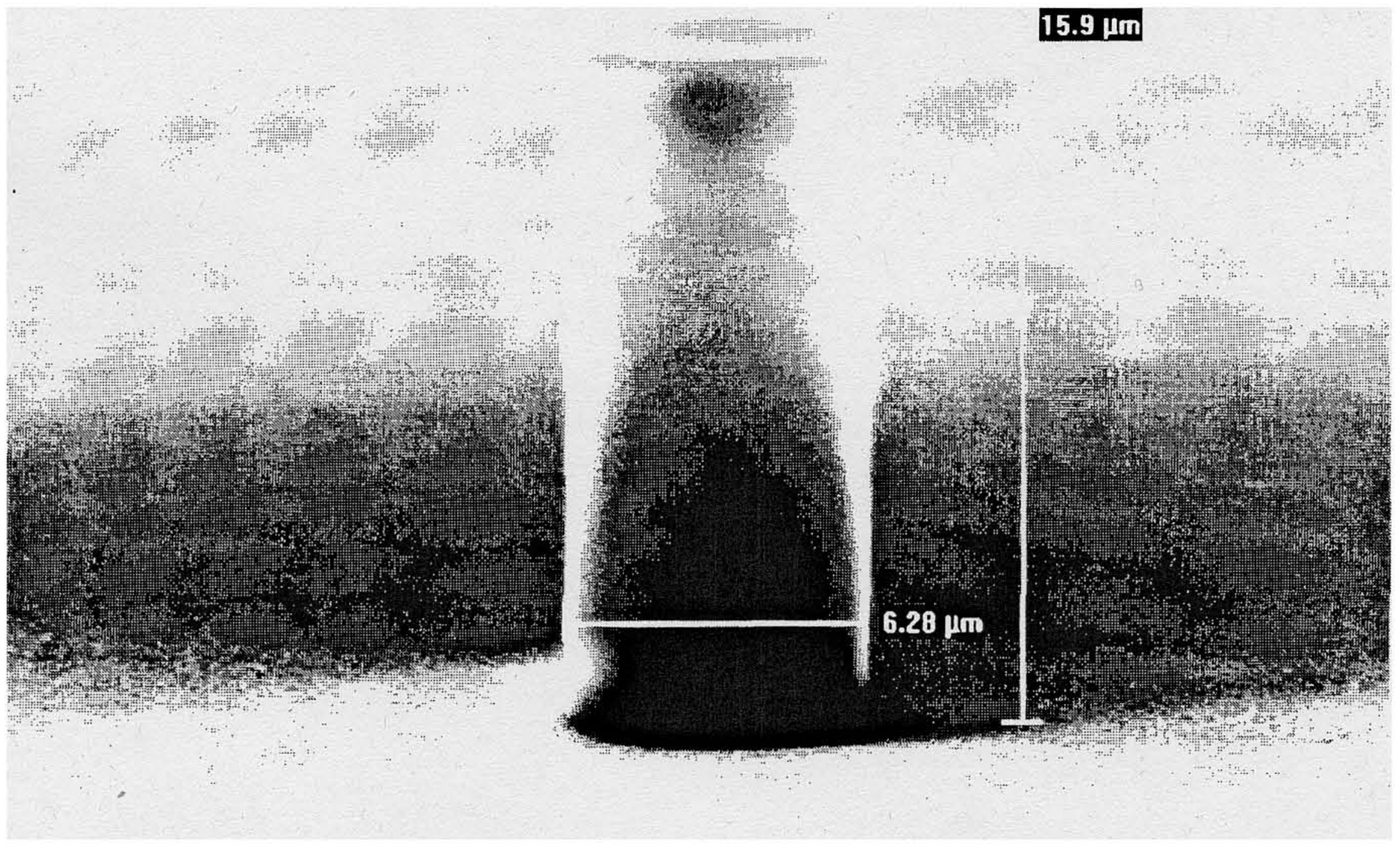

Fig. 5. SEM side view of a micropipette. This micropipette was coated with an additional 200 nm CVD SiO_2 layer that explains the smoothness of the top edges.

3. Results

3.1. Realization of the microsystem

Fig. 4 shows a SEM photograph of a realized micropipette. The 500-nm thick silicon nitride micropipette wall is perfectly shaped and is reproducible at the wafer level. The reactive ion etching process used to remove the nitride layer is directional enough, so that it perfectly etches the top of the micropipette, whereas the vertical walls are preserved (Fig. 5). The length of the protruding part of the micropipettes is about 16 µm. As they are partly embedded in the silicon, their overall length is 50 µm. The micropipettes are separated by 150 µm and the overall area of the array is 0.4 mm^2 (Fig. 6).

The advantage of using DRIE technology, instead of KOH etching, to form the micropipette is its excellent control of the openings (inner diameter) of the capillaries. Indeed, this technology is quite independent on the etching depth. In addition, the protruding height of the micropipettes is well

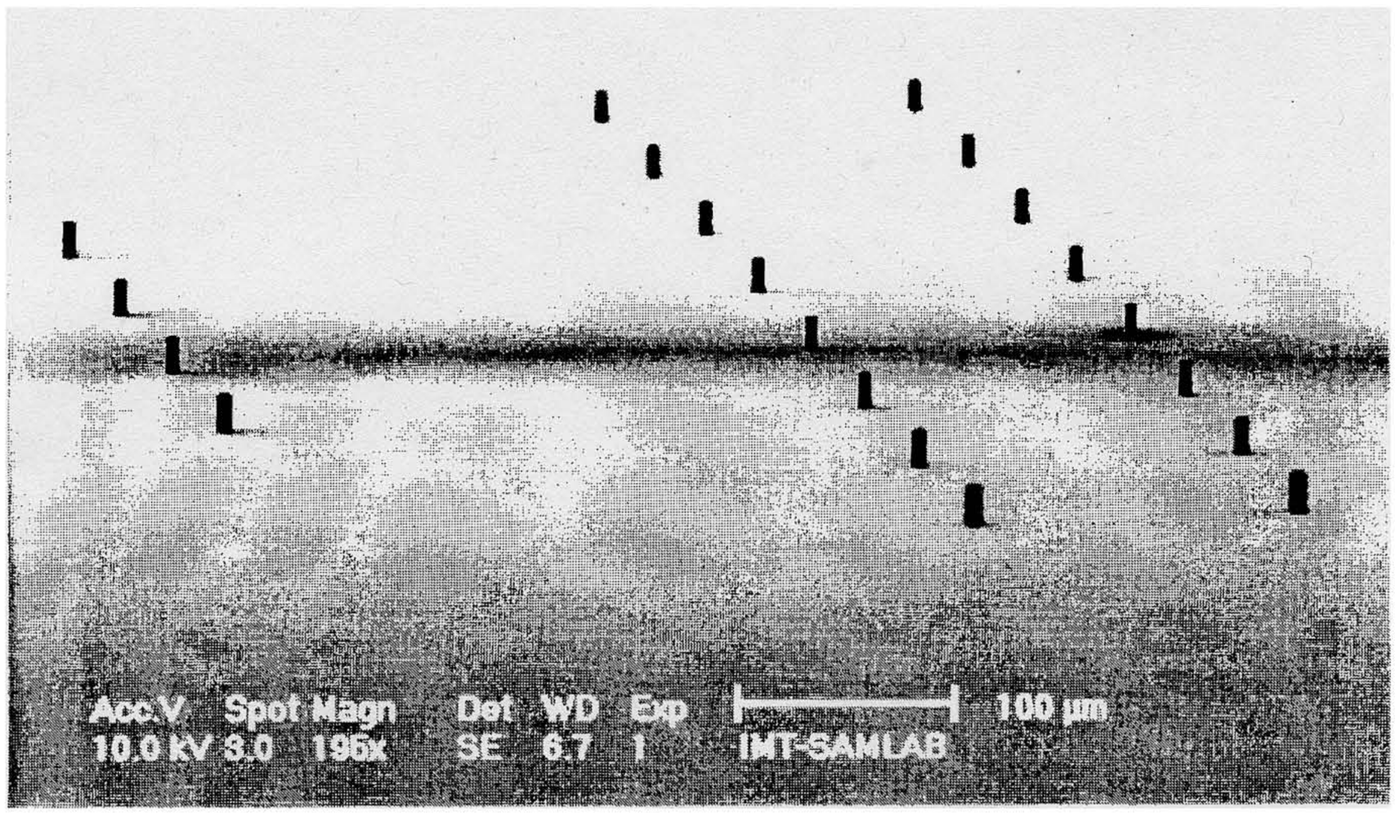

Fig. 6. Partial view (SEM) of an array of micropipettes, separated by 150 µm (the array is tilted at 80°).

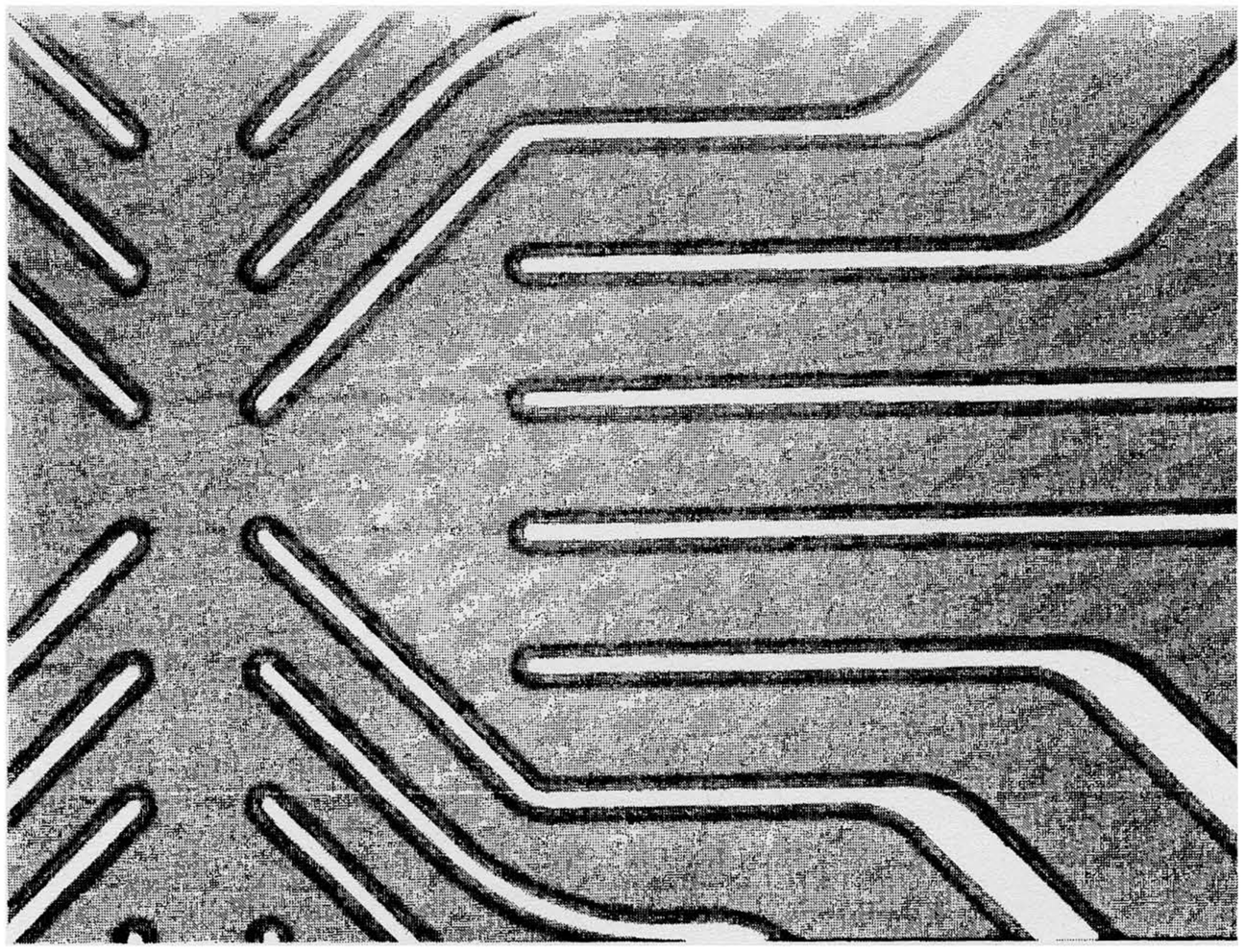

Fig. 7. Picture of the 20-μm deep and 50-μm wide microchannels (dimensions at the end of the channels), with a 15-μm wide Pt layer patterned at the bottom of each microchannel.

determined by controlling the etching time in KOH solution. At this point, the height of the micropipettes should be chosen as a function of the target cell size.

Fig. 7 shows the network of the microchannels with their metallic electrodes patterned at the bottom of the channels. Despite the depth of the channels (20 μm), the lift-off process turned out well and electrodes as small as 10 μm could be patterned. The 90-μm wide channel-inlet, that widens into a much larger channel (290 μm), is shown in Fig. 8. It allows the dispensing of the ion-selective cocktail.

The ready-to-use device is presented in Fig. 9. A glass reservoir is glued on the silicon chip, in the middle of which the cavity containing the micropipettes array is located. The inlets for the membrane filling can be seen on both side of the silicon chip. The overall dimensions of the chip are 18 mm × 20 mm.

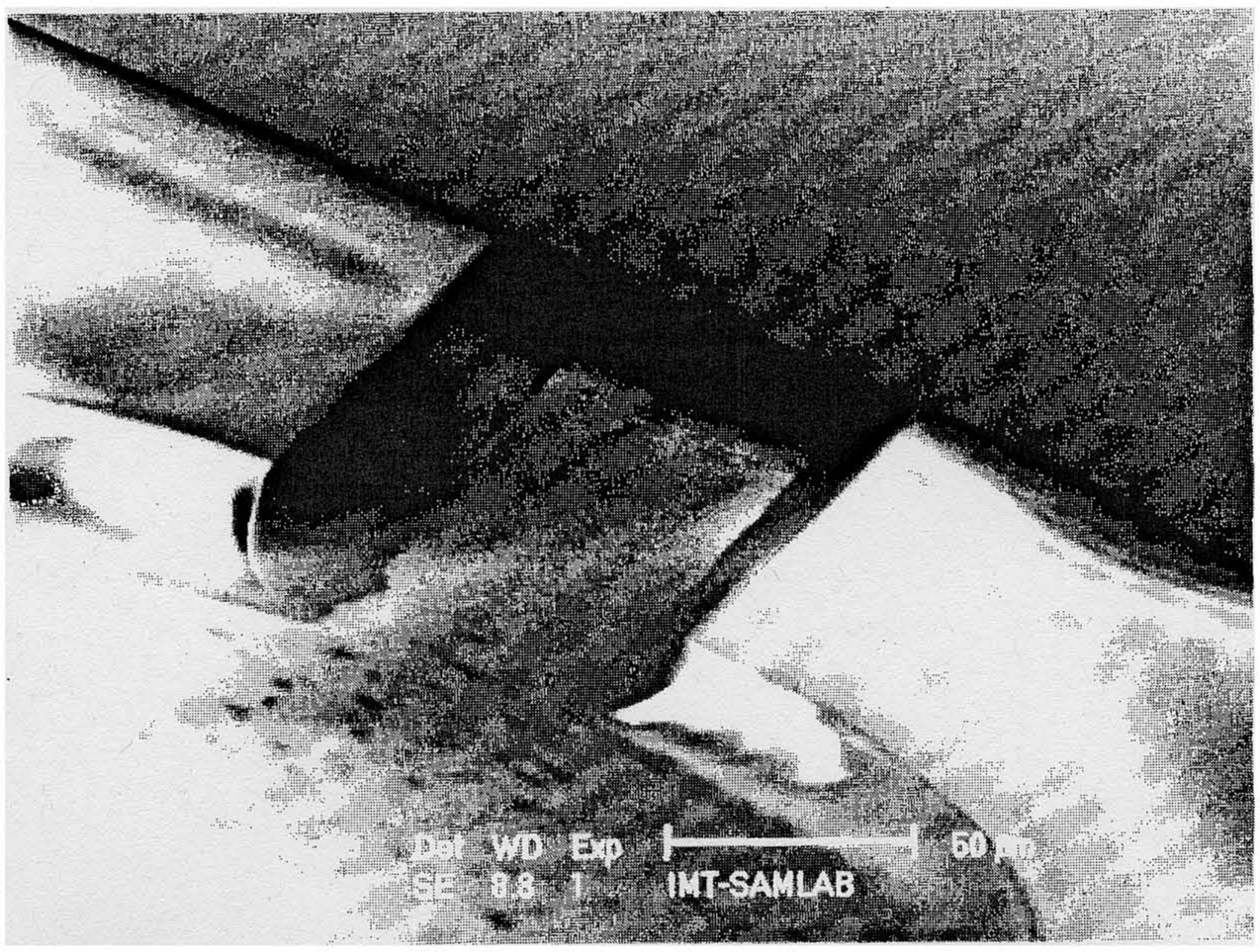

Fig. 8. SEM picture of the inlet of a microchannel for the filling of the ion-selective membrane. A Pt electrode is visible at the bottom of the channel.

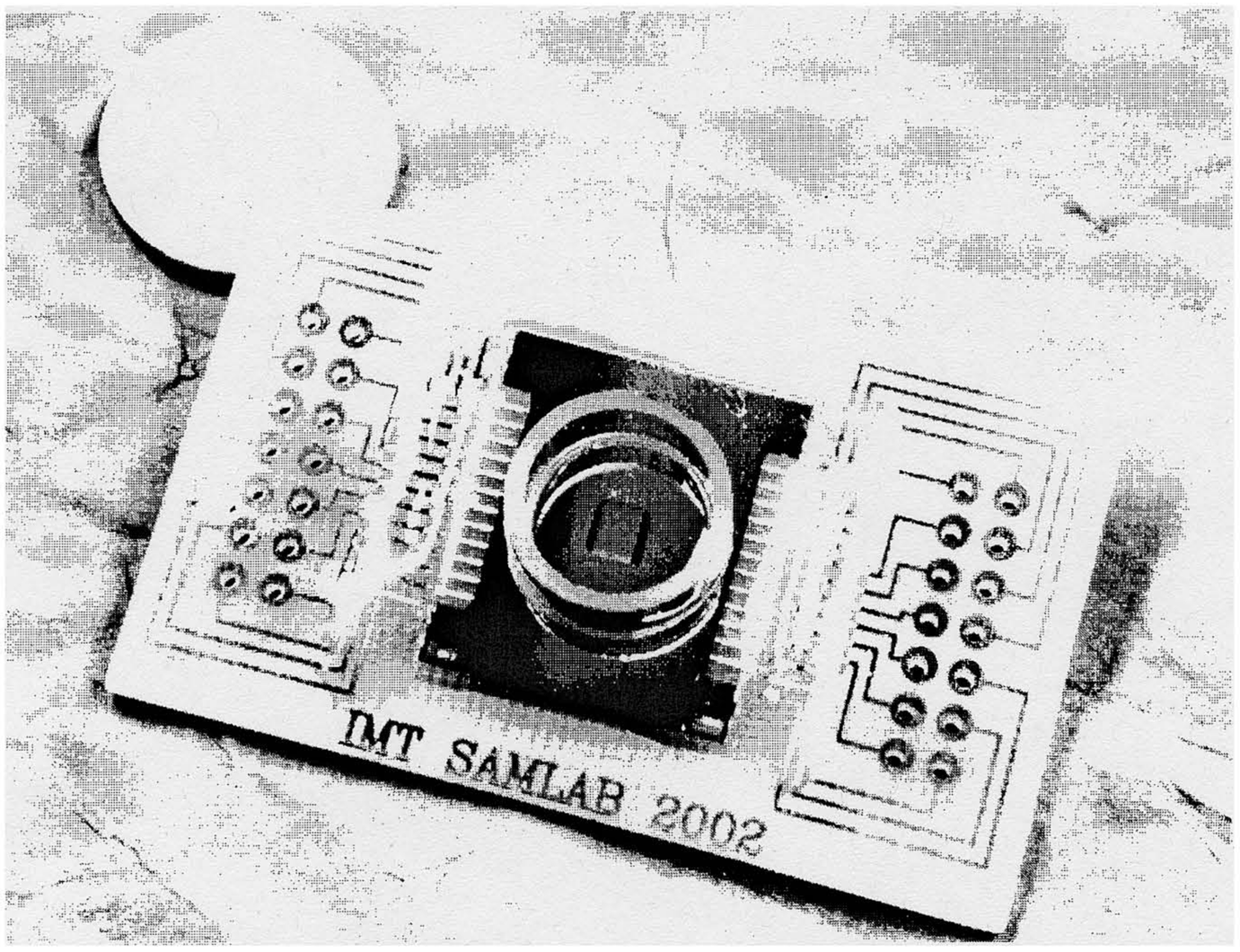

Fig. 9. Photograph of the entire device mounted on a PCB. A glass reservoir is glued on top of the chip.

3.2. Filling of the micropipettes with the ion-selective cocktail

Before filling the micropipettes with the membrane, the device was dehydrated at 90 °C in a convection oven for 24 h and then silanized to improve the adhesion of the membrane to the nitride inner walls. A vapor phase silanization was carried out by using *N,N*-dimethyltrimethylsilylamine (purum, Fluka), during 60 min.

The ion-selective membrane cocktail was prepared with the following components: 4.2 wt.% ETH 129 (calcium ionophore II), 0.8 wt.% sodium tetraphenylborate, 80.2 wt.% 2-nitrophenyl octyl ether, 14.7 wt.% poly(vinyl chloride) high molecular weight [15,16]. The membrane components

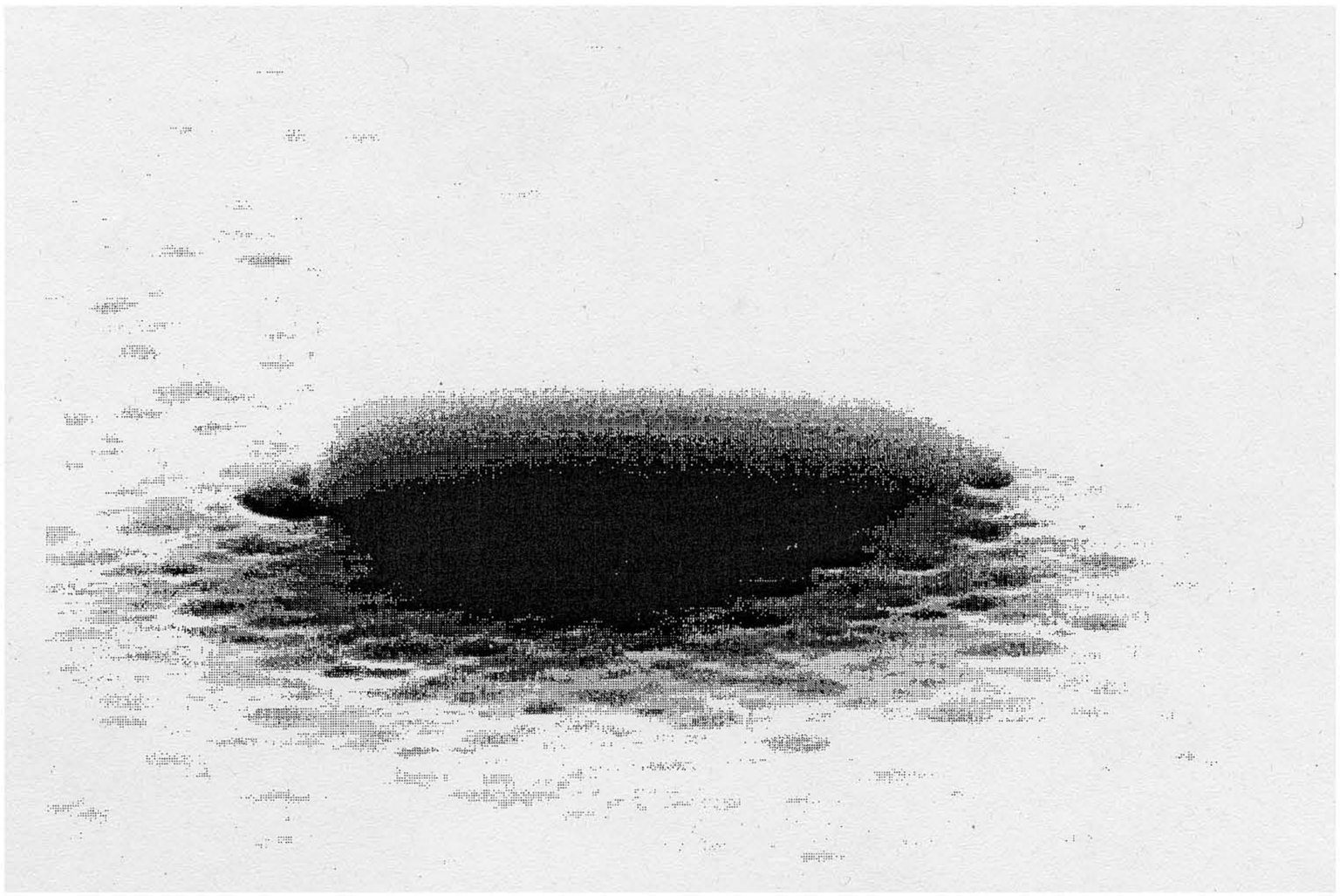

Fig. 10. ESEM pictures of a micropipette (tilted at 75°) filled with the membrane cocktail. Due to the high surface tension, the membrane does not overflow.

(244 mg) were dissolved in 0.4 ml cyclohexanone (all Selectophore, Fluka). Cyclohexanone was preferred to THF, which is generally used for microelectrodes applications, since it has a lower evaporation rate.

Following a partial evaporation of cyclohexanone to slightly thicken the mixture [16], the ion-selective membrane was dispensed in the microchannels with a syringe. The membrane was dried for 24 h under ambient conditions. The observation of the filled micropipettes in an environmental scanning electronic microscope (ESEM) (Fig. 10), showed that the surface tension at the top of the micropipette, was high enough, so that the membrane did not overflow. Then, the reservoir was filled with a 10^{-4} M $CaCl_2$ solution (p.a., Merck) and electrodes preconditioned during 24 h.

3.3. Ca^{2+} calibration curve

The measurements were carried out with a flexible mini-Ag/AgCl/3 M KCl reference electrode (DRIREF-2, World Precision Instruments) and a home-made high impedance (10^{14} Ω) input amplifier. Calcium buffers (CALBUF-1, [17]) purchased from WPI were used for the calibration tests. A typical example of the Ca^{2+} response from one microelectrode is presented in Fig. 11. It can be seen that the linear region extends from 10^{-6} M to at least 10^{-1} M with a slope of 27 mV/decade. It should be mentioned that the responses were measured 10 s after the filling of the sample solutions in the reservoir. This seems to indicate that the response time of the sensor is rather fast.

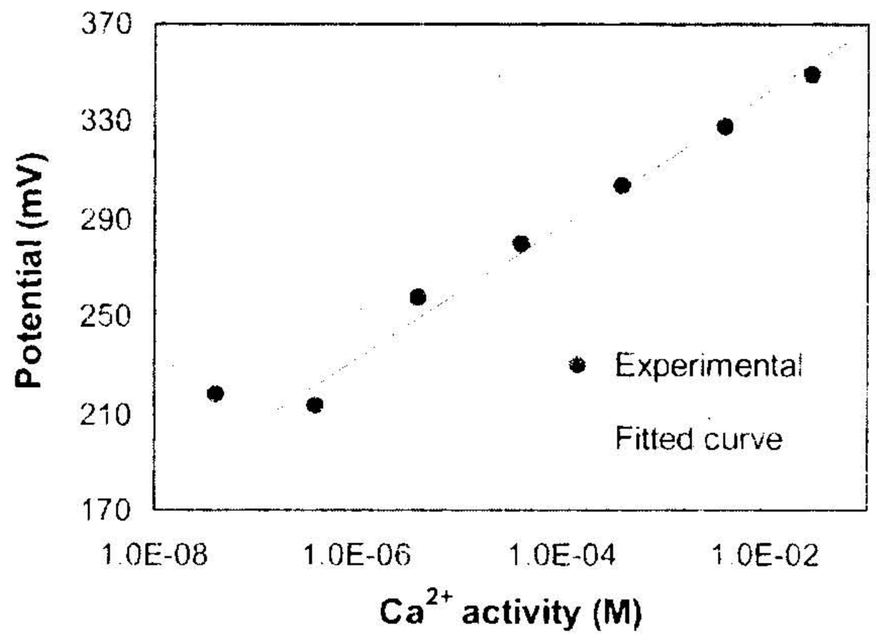

Fig. 11. A typical response of a Ca^{2+} calibration curve. Calcium buffers were used for these measurements, that were recorded 10 s after the filling of the sample solutions.

3.4. Biocompatibility of the microsystem

A cell culture experiment was performed in order to check the biocompatibility of the microsystem. Prior to the cell culture, the device was UV exposed for 30 min for sterilization, then the physiological medium was added in the reservoir as well as the cells. HepG2 human hepatocellular cell line were used [18]. After 48-h incubation, the cells have proliferated and grown to confluency on the array of microelectrodes indicating that array is not toxic and thus biocompatible for cells in culture. Fig. 12 shows a picture of the cell culture next to several micropipettes.

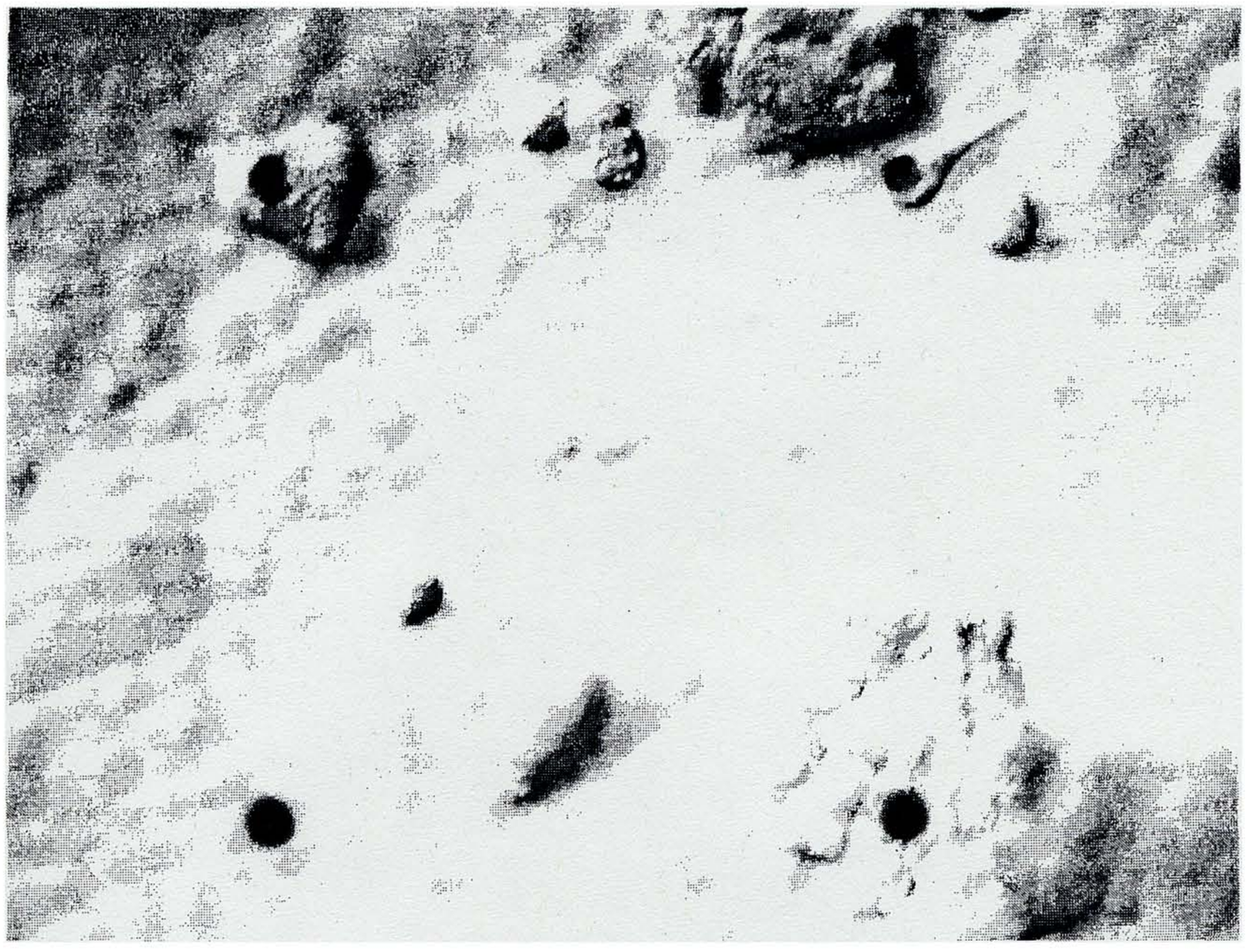

Fig. 12. HepG2 human hepatocellular cells after 48-h incubation. The arrow shows a 5 μm inner diameter micropipette in the middle of an hepatocyte.

4. Discussion and conclusion

In this paper, we reported the design and the fabrication of an array of ion-selective microelectrodes, with inner and outer diameters of 5 and 6 μm, respectively. The microelectrodes were made of silicon nitride micropipettes that were filled with an ion-selective membrane based on neutral ionophore ETH 129. These micropipettes could be made in a very reproducible manner and their filling with a viscous ion-selective cocktail containing 14% PVC could be achieved by capillary forces. The established technology is currently used for the fabrication of an array of microelectrodes with smaller dimensions (1.7 μm inner diameter) that are more appropriate for hepatocyte intracellular recording.

Preliminary measurements in calcium buffered solutions were realized and very promising results were achieved. For solutions with Ca^{2+} activities ranging from 10^{-6} to 10^{-1} M Ca^{2+} a nearly Nernstian response behavior was obtained. Further measurements intended to increase the concentration range and to define the selectivity and the response time of the sensors are in progress.

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References

[1] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21.

[2] S. Baudet, L. Hove-Madsen, D. Bers, Methods in cell biology, in: R. Nuccitelli (Ed.), A Practical Guide to the Study of Calcium in Living Cells, vol. 40, Academic Press, 1994.

[3] R.P. Haugland, I.D. Johnson, Intracellular ion indicators, in: W.T. Mason (Ed.), Fluorescent and Luminescent Probes for Biological Activity, 1999, pp. 40–50.

[4] D. Ammann, Ion-selective microelectrodes, in: Principles, Design and Applications, Springer-Verlag, Berlin, 1986.

[5] R.D. Purves, Microelectrodes Methods for Intracellular Recording and Ionophoresis, Academic Press, London, 1981.

[6] A. Takahashi, P. Camacho, J. Lechleiter, B. Herman, Measurements of intracellular calcium, Physiol. Rev. 79 (4) (1999).

[7] N. Fertig, C. Meyer, R. Blick, C. Trautmann, J. Behrends, Microstructured glass chip for ion channel electrophysiology, Phys. Rev. E 64 (4) 2001.

[8] Aviva Biosciences USA, available at: http://www.avivabio.com/.

[9] S. Pedersen, J. Kutschinsky, S. Friis, K. Krzywkowski, C.L. Tracy, R. Vestergaard, C. Sorensen, H. Vennerberg, R. Taboryski, An electrophysiological lab-on-chip, Proc. Transducers, Boston, 2003, p. 1059.

[10] T. Lehnert, M. Gijs, R. Netzer, U. Bischoff, Realization of hollow SiO_2 micronozzles for electrical measurements on living cells, Appl. Phys. Lett. 81 (26) (2002) 5063–5065.

[11] A. Uhlig, E. Lindner, C. Teutloff, U. Schnakenberg, R. Hintsche, Miniaturized ion-selective chip electrode for sensor application, Anal. Chem. 69 (1997) 4032–4038.

[12] V.V. Cosofret, M. Erdösy, T.A. Johnson, R.P. Buck, R.B. Ash, M.R. Neuman, Microfabricated sensor arrays sensitive to pH and K^+ for ionic distribution measurements in the beating heart, Anal. Chem. 67 (1995) 1647–1653.

[13] K. Chun, G. Hashiguchi, H. Toshiyoshi, H. Fujita, Fabrication of array of hollow microcapillaries used for injection of genetic materials into animal/plant cells, Jpn. J. Appl. Phys. 38 (Part 2, no. 3A) (1999) L279–L281.

[14] P. Luginbuhl, P.F. Indermuhle, M.A. Grétillat, F. Willemin, N.F. de Rooij, D. Gerber, G. Gervasio, J.L. Vuilleumier, D. Twerenbold, M. Düggelin, D. Mathys, R. Guggenheim, Femtoliter Injector for DNA mass spectrometry, Sens. Actuators B 63 (2000) 127–177.

[15] D. Ammann, T. Bührer, U. Schefer, M. Müller, W. Simon, Intracellular neutral carrier-based Ca^{2+} microelectrode with submanomolar detection limit, Pflügers Arch. 409 (1987) 223–228.

[16] S. Baudet, L. Hove-Madsen, D.M. Bers, How to make and use calcium-specific mini and microelectrodes, in: R. Nuccitelli (Ed.), Methods in Cell Biology, vol. 40, 1994 (Chapter 4).

[17] R.Y. Tsien, T.J. Rink, Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium, Biochim. Biophys. Acta 599 (1980) 623–638.

[18] D.P. Aden, A. Fogel, S. Plotkin, I. Damjanov, B.B. Knowles, Controlled synthesis of HbsAg in a differentiated human liver carcinoma derived line, Nature 282 (1979) 615–616.

Biographies

Olivier T. Guenat received his Microtechnology degree from the University of Applied Sciences (HES) of Bienne, Switzerland, in 1990, and his M.Sc. degree in Physics and Electronics from the University of Neuchâtel, Switzerland in 1995. For half a year, he worked in the microfluidic field (microvalves and micropumps) at Molecular Devices Corp., Sunnyvale, CA. In November 1995, he joined the Institute of Microtechnology (IMT) of the University of Neuchâtel, where he got the Ph.D. degree for his works related to coulometric and volumetric nanotitrations, as well as for his studies about indirect electroosmotic pumping. Since 2000, he is working as a post-doc in the field of bio-electrochemical sensors. His main research interests include BioMEMS as well as sensors for the detection of trace metals in water.

Jean-François Dufour received his Mathematical degree from the University of Geneva, Switzerland, in 1984, and his Medical degree from the same University 2 years later. After 3 years of training in internal medicine in Geneva he worked from 1989 to 1990 at the Department of Clinical Pharmacology in Bern, Switzerland. He then moved to the Physiology Department of Tufts University in Boston where he researched on calcium signaling in liver cells. From 1995 to 1997, he completed his training in gastroenterology at the New England Medical Center in Boston. In 1997, he returned to the Department of Clinical Pharmacology at the University of Bern where he is participating in the clinical activities of the liver center and leads a group of research on signaling in liver cells. He is a member of the scientific committee of the European Association for the Study of the Liver.

Peter D. van der Wal received an engineering degree in Chemical Technology in 1985 and his Ph.D. in natural sciences in 1991, both from the University of Twente, The Netherlands. During the years 1991–1992, he participated in a project entitled "ion sensors for horticulture," a collaboration between Priva B.V., Twente Technology Transfer and the Organic Chemistry group of the University of Twente. In 1993, he joined the "Sensors, Actuators and Microsystems Laboratory" (SAMLAB) group of the Institute of Microtechnology in Neuchâtel, Switzerland as a scientific

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Werner E. Morf graduated in chemistry in 1969, and received his Ph.D. degree (Dr. sc. techn.) in 1972 from the Swiss Federal Institute of Technology (ETH), Zürich. He remained at ETH Zürich as a senior scientist, and obtained the venia legendi as lecturer (Privat-dozent) in 1980. From 1990 to 1993, he held an industrial R + D position. In 1994, he joined the Institute of Microtechnology (IMT) of the University of Neuchâtel, Switzerland. His activities include chemical sensors, electrochemistry, and microsystems. He was recipient of the Swiss Chemists' Association Award in 1975.

Nicolaas F. de Rooij received an M.Sc. degree in physical chemistry from the State University of Utrecht, The Netherlands, in 1975, and a Ph.D. degree from Twente University of Technology, The Netherlands, in 1978. From 1978 to 1982, he worked at the Research and Development Department of Cordis Europa N.V., The Netherlands. In 1982, he joined the Institute of Microtechnology of the University of Neuchâtel, Switzerland (IMT UNI-NE), as professor and head of the Sensors, Actuators and Microsystems Laboratory. Since October 1990 till October 1996 and again from October 2002, he is acting as director of the IMT UNI-NE. He lectured at the Swiss Federal Institute of Technology, Zurich (ETHZ), and since 1989, he has been a part-time professor at the Swiss Federal Institute of Technology, Lausanne (EPFL). His research activities include microfabricated sensors, actuators, and microsystems. He was a member of the steering committee of the International Conference on Solid-State Sensors and Actuators and of Eurosensors. He acted as European Program Chairman of Transducers '87 and General Chairman of Transducers '89. He is a member of the editorial boards for the journals *Sensors and Actuators, Sensors and Materials* and *the IEEE Journal of Microelectromechanical Systems*. Dr. de Rooij is an IEEE Fellow.

Milena Koudelka Hep received a B.Sc. in chemistry in 1974 and a Ph.D. in 1978 at the University of Geneva. After a 4-year position of a maître-assistant also at the University of Geneva, she worked as a senior research assistant in the chemical sensor group of the Swiss Center for Electronics and Microtechnology. Since mid-1986, she has been working at the Institute of Microtechnology of the University of Neuchâtel in the "Sensors, Actuators and Microsystems" group. Currently associate Professor, she is heading the activities in the field of (bio)electrochemistry.

Her main research interests are electrochemical sensors, biosensors and microelectrode arrays for environmental and biomedical applications.