



Modification of standard CMOS technology for cell-based biosensors

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ABSTRACT

We present an electrode based on complementary metal oxide semiconductor (CMOS) technology that can be made fully biocompatible and chemically inert using a simple, low-cost and non-specialised process. Since these devices are based on ubiquitous CMOS technology, the integrated circuits can be readily developed to include appropriate amplifiers, filters and wireless subsystems, thus reducing the complexity and cost of external systems. The unprocessed CMOS aluminium electrodes are modified using anodisation and plating techniques which do not require intricate and expensive semiconductor processing equipment and can be performed on the bench-top as a clean-room environment is not required. The resulting transducers are able to detect both the fast electrical activity of neurons and the slow changes in impedance of growing and dividing cells. By using standard semiconductor fabrication techniques and well-established technologies, the approach can form the basis of cell-based biosensors and transducers for high throughput drug discovery assays, neuroprosthetics and as a basic research tool in biosciences. The technology is equally applicable to other biosensors that require noble metal or nanoporous microelectrodes.

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1. Introduction

The direct interfacing of semiconductor technology to live, excitable biological tissue was initially demonstrated in the late 20th century (Fromherz et al., 1991) and has continued to be of significant interest for applications such as bidirectional stimulation and recording systems (Supplementary Information Table S1), high content screening or implantable electronic devices. The electrical interface between biological tissue and semiconductor electronics is a vital component of a successfully functioning system and often takes the form of a low impedance electrode (Graham et al., 2011a; Hierlemann et al., 2011). The electrode area of the semiconductor die must be both biocompatible and chemically inert which has led to the use of existing manufacturing methods requiring specialist and expensive device manufacture. This has limited their application in key areas such as high-throughput screening and drug discovery.

This article presents an economical, flexible and biocompatible bio-sensing device based on standard CMOS technology in which aluminium electrodes are modified using simple anodisation and plating techniques. The modification process does not require

intricate and expensive semiconductor post-processing equipment and can be performed with simple bench-top equipment. A clean-room environment is *not* required. This new approach provides the basis for low-cost mass-manufacture of electrodes. We demonstrate that the modified CMOS is readily configured for neuron action potential recordings and cell-substrate impedance systems and such an approach can be used in many other applications such as DNA hybridisation (electrophoresis) (Liu et al., 2006) and DNA sequencing (Ion Torrent Systems Inc., 2010). Individual microelectrodes are addressable using on-chip circuits and can meet the requirements for long-term and non-invasive cell culture assays and label-free high content screening.

2. Materials and methods

2.1. CMOS device construction

The CMOS integrated circuits (ICs) comprised of 48 electrodes for recording extracellular action potential activity in adult mammalian neurons and for sensing the electric cell-substrate impedance (ECIS) of an epithelial cell line.

The CMOS ICs were fabricated by austriamicrosystems AG, Germany using 0.8 μm technology. Thirty devices were supplied in 48-pin ceramic dual in line (DIL) packages with removable die-cavity lids. A further 18 bare dice were partially encapsulated

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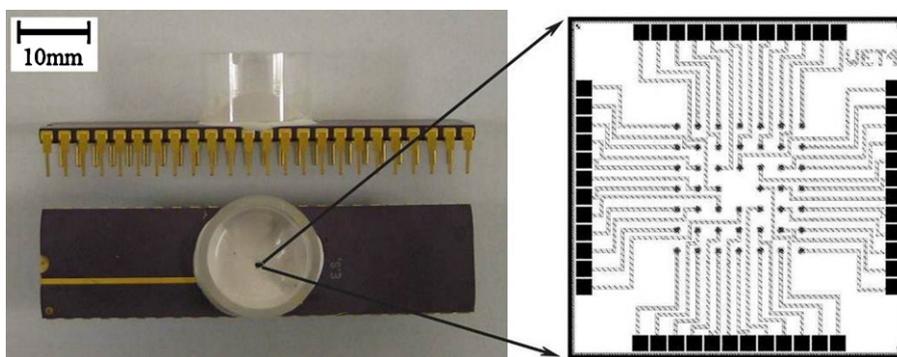


Fig. 1. The CMOS device and electrode layout. The silicon dimensions are 3.1 mm × 3.1 mm.

by Quik-Pak (San Diego, U.S.) using their standard manufacturing process, except that biocompatible compounds were specified, namely: Silastic Medical Adhesive Silicone Type A (Dow Corning, U.S.) for placement of the window frame and Hysol CB064 (Loctite, U.S.) for encapsulation (Graham et al., 2011b). A cyanoacrylate adhesive was used for a permanent bond, or the glass adhered using Silastic 9161 (Dow Corning, UK) so that the packages could be more easily disassembled, e.g. for scanning electron microscopy analysis.

Glass cylinders, 10 mm tall, 13 mm internal diameter (QB Glass, UK), were adhered to the upper surface of the packages encircling the open die cavities so that the 48 electrodes were exposed at the base of each chamber. These formed receptacles for further processing of the electrodes and subsequently functioned as cell culture chambers (Fig. 1).

To interface the CMOS technology directly with biological tissue, while maintaining biocompatibility and chemical inertness the aluminium electrodes were anodised to form nano-porous alumina (aluminium oxide). The nano-pores were subsequently infiltrated with gold and plated (Graham et al., 2009a,b, 2010) with platinum black to reduce and optimise the electrode electrical impedance for neuronal recordings (Fig. 2). This economic and simple CMOS modification process begins with anodisation of the aluminium electrodes for approximately 40 min in 0.4 M phosphoric acid, followed by 20 min plating of the anodised nano-porous alumina in 59 mM gold chloride ($\text{HAuCl}_4\cdot 3\text{H}_2\text{O}$) and approximately

one minute for platinum black deposition using chloroplatinic acid ($\text{H}_2\text{PtCl}_6\cdot 6\text{H}_2\text{O}$) with 264 μM Lead(II) acetate trihydrate. Fig. 2 (supported by Supplementary Fig. S1) shows a section of an electrode processed using this method, illustrating the uniform anodisation and gold deposition across the area of the electrode. Full details of the method were presented in Graham et al. (2010). The net result of this process is that the CMOS aluminium electrode, which normally corrodes when in contact with a saline culture medium, is converted to a chemically inert, low impedance electrode consisting of aluminium oxide, gold and high surface area platinum black.

2.2. Cell culture

Rat dorsal root ganglion cells were cultured as previously described (England et al., 2001). In brief, adult (250 g) Sprague-Dawley rats were housed and killed according to UK Home Office regulations. The dorsal root ganglia were dissected into Ham's F14 media supplemented with penicillin (100 IU ml^{-1} Sigma, UK), streptomycin (100 $\mu\text{g ml}^{-1}$, Sigma) L-glutamine (2 mM, Sigma) and 10% foetal calf serum (FCS, Invitrogen, UK). The ganglia were transferred to F14 medium containing 0.125% collagenase and incubated at 37 °C in 5% CO_2 95% air for 40 min. The cells were further transferred into F14 medium with added nerve growth factor (50 ng ml^{-1}) and plated on the sterile CMOS devices which had been previously coated with poly-L-lysine (15–30 kDa, 0.01%, w/v). The devices were then kept at 37 °C in 5% CO_2 /95% air for 18–24 h.

The Caco-2 human epithelial cell line was chosen for the ECIS recordings as the cells continually divide to produce a monolayer (Hidalgo et al., 1989). Cells were cultured in DMEM/F-12 media supplemented with L-glutamine (1%, w/v, Sigma), FCS (10%, w/v) and non-essential amino acids (1%, w/v). Cells were grown in 50 ml flasks (Nunc, UK) to 90–100% confluency at 5% CO_2 and 37 °C. The cells were dissociated from the flasks by 3–6 min trypsinisation (0.5%, w/v, Worthington) after having been rinsed in EDTA (10 mM, Sigma, UK) and calcium and magnesium free phosphate buffered saline. Detached cells were spun down (5 min at 700 rpm), re-suspended in fresh medium and plated onto the sterilised (ethanol 30 min) devices.

2.3. Electrophysiological recording

To demonstrate that the simply modified CMOS can sense the fast electrical activity of neurons, primary cultures of rat dorsal root ganglion (DRG) cells (England et al., 2001) were plated onto poly-lysine coated CMOS devices that were incubated for 18 h, as described in the previous section. Recordings were undertaken at 21 °C by placing the CMOS devices in a 48-pin zero insertion

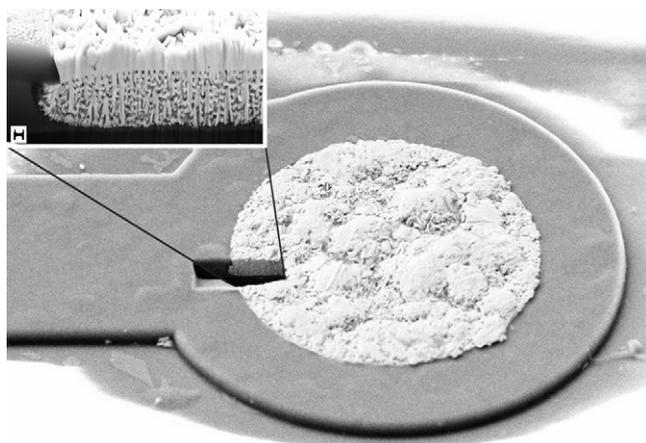


Fig. 2. Scanning electron microscope (SEM) image of a single electrode with section created by milling with a focused ion beam (FIB). The inner circular area is the electrode (30 μm diameter). This is surrounded by approximately 10 μm of aluminium which appears as an outer 'ring' which is connected to the aluminium track leading off to the left. The inset shows the porous alumina filled with gold. Inset scale bar 200 nm.

force (ZIF) socket connected to a 64 channel preamplifier (PAG64, Multichannel Systems) which was housed in a Faraday cage. The preamplifier was connected to an MC card (Multichannel Systems) located in a PC (generic) running MC Rack software (v 3.1, Multichannel Systems). The sample rate was 25 kHz per channel and the recording bandwidth was 1 Hz to 5 kHz. Spontaneous activity was continually recorded onto a hard disk, initially in the culture medium and then in a modified Krebs's solution (mM): NaCl (130), KCl (3), MgCl₂ (1), CaCl₂ (1), HEPES (5), Glucose (11), pH adjusted to 7.4 using 2 M NaOH. Tetrodotoxin (100 nM, TTX, Tocris, UK), Adenosine triphosphate (100 μM, ATP, Sigma, UK) and KCl (12 mM) were applied via the superfusion system at 21 °C flowing at 2.3 ml min⁻¹.

2.4. Electric cell-substrate impedance sensing recordings

The ability of the CMOS device to function as an electric cell-substrate impedance sensor and monitor the real-time electrical impedance changes associated with cell growth and division (Hidalgo et al., 1989; Prakash & Abshire, 2008) was assessed using the Caco-2 cell line.

CMOS devices were placed in an incubator (5% CO₂/95% air, 37 °C) mounted in a 48-pin ZIF socket. The 48 electrodes on a CMOS device were connected together as the active electrode and a reference electrode (Ag/AgCl wire) was placed in the culture media bathing the cells. Impedance was measured using a precision impedance analyser (Agilent 4294A) at the frequency range 40 Hz to 1 MHz. Samples were taken every 15 s for the first 15 min then every 1 min for a further 45 min and finally every 60 min for up to 5 days. Control devices with no cells showed impedance changes over the same period of less than 200 Ω.

2.5. Data analysis

For the electrophysiological recordings, data stored on the hard disk were converted to pClamp ABF files by MCDDataTool (Multichannel Systems) and spike frequency and fast Fourier analysis data were generated using the pClamp (v8.2, Axon Instruments, USA) program suite. For the ECIS experiments, the imaginary impedance Z'' was calculated using the equation $Z'' = 1/(2\pi fC)$, where C is the capacitance, f the signal frequency (47.9 kHz) and Z the complex impedance. Statistical analysis was performed using ANOVA with $p < 0.05$ considered significant.

3. Results

3.1. Neuronal recordings

Fig. 3 shows successful recordings of action potentials initially performed in the culture medium and the inset shows an individual spiking event. The spontaneous activity of a cell, measured over a 20 s epoch, was 14.7 ± 0.1 Hz (mean \pm SEM, $n = 20$). The signal to noise ratio was approximately 5:1 and power spectral analysis of the noise showed no unexpected peaks (Supplementary Information Fig. S2).

On superfusion with a buffer solution the firing frequency was 17.4 ± 0.1 Hz ($n = 20$). In order to confirm that the fast spiking activity was generated by action potentials of neurons they were successfully blocked in a selective and reversible manner by 100 nM tetrodotoxin, a potent selective sodium channel blocker (Fig. 4). Fig. 5 shows that an increase in extracellular potassium from 3 mM to 15 mM to depolarise the cells (by ~ 40 mV, predicted from the Nernst equation) also led to a reversible and significant ($p < 0.01$) increase in neuron firing rate. Similarly, the application of 100 μM adenosine triphosphate, an excitatory neurotransmitter, also significantly ($p < 0.01$) increased the activity of the cell.

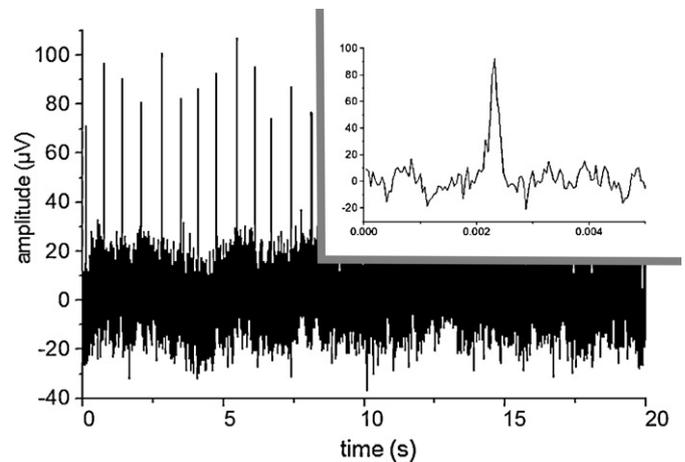


Fig. 3. Extracellular spontaneous action potentials (spikes) recorded from a DRG sampled at 25 kHz (bandwidth 1–5000 Hz). The frequency of the neuron activity, measured over a 20 s epoch, was 14.7 ± 0.1 Hz (mean \pm SEM, $n = 20$) and the signal to noise ratio was approximately 5:1. The inset shows one of the spikes on an expanded time axis.

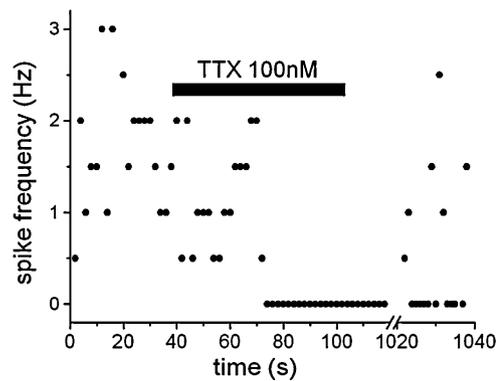


Fig. 4. The potent and selective sodium channel blocker, tetrodotoxin, reversibly inhibits spontaneous action potential activity. Spike frequency plotted in 1 s epochs before, during (black bar) and after the application of 100 nM tetrodotoxin (TTX). The gap in the horizontal axis is 900 s. Spike frequency is measured as 'spikes per second' – Hertz.

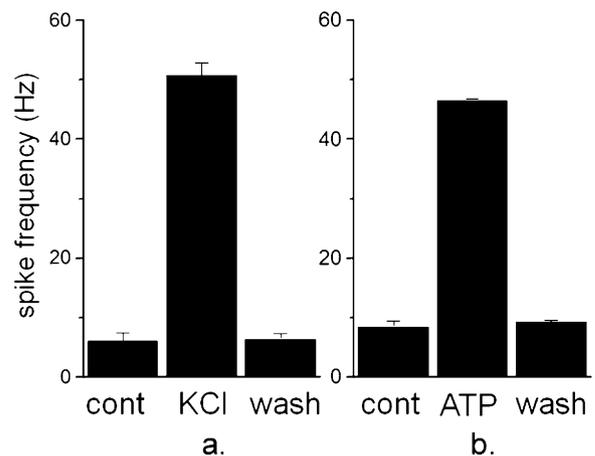


Fig. 5. DRG excitability can be increased by extracellular potassium and neurotransmitters. (a) Mean (\pm SEM, 15×1 s epochs) spike frequency before ('cont'), during ('KCl') an after ('wash') an increase in [K⁺]_o (3–15 mM) and (b) before ('cont'), during ('ATP') and after ('wash') application of 100 μM adenosine triphosphate.

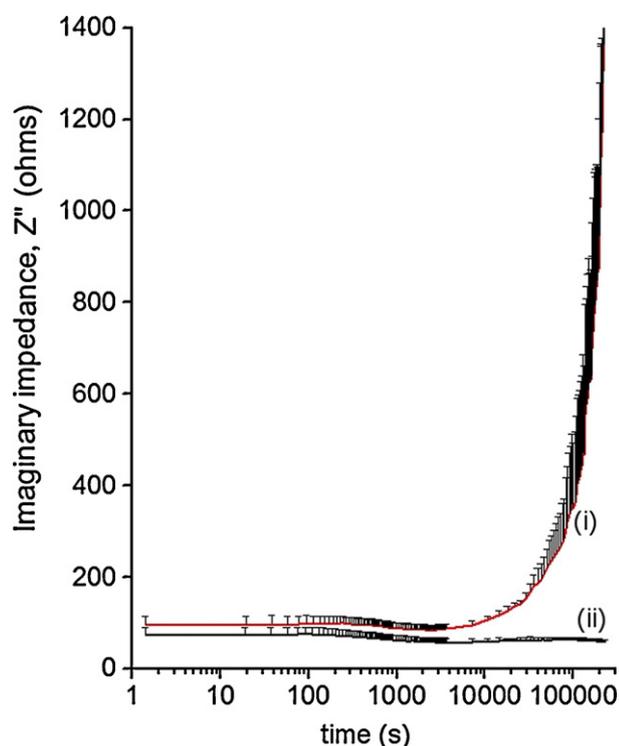


Fig. 6. Cell growth can be measured by impedance changes. Mean imaginary impedance, Z'' (measured at 47.9 kHz at 37 °C) over 2.7 days. (i) 4 devices containing Caco-2 cells and (ii) 3 devices containing the same culture medium but no cells. Only positive error bars (SEM) are shown for clarity.

3.2. ECIS recordings

Fig. 6 illustrates the measured increase in electrical impedance with time as the cells form a monolayer over the modified electrodes, compared to CMOS devices under identical conditions but containing no cells.

4. Discussion

The neuronal recording results demonstrate that the simply modified CMOS device is able to record non-invasively both the spontaneous and evoked extracellular action potentials of mammalian neurons over a period of hours. Therefore, the device can be used to monitor the excitability changes in cells in response to both physiological and pharmacological interventions. In view of the stability of the device (see Fig. 6), long-term monitoring of activity is possible for many days, probably weeks, making it suitable for in vitro investigations into drug dependence, complex network activity, neurodegeneration and neuronal development.

The ECIS results illustrate the flexibility of the modified CMOS technology. In this configuration, the electrode array can be used to study tumour growth, wound healing, cell migration and for understanding how cells interact with a substrate (Bionas GmbH, 2010). A model is presented in Wegener et al. (2000) which shows an almost linear relationship between capacitance and the fraction of electrode surface covered by cells: with 1.0 being the normalised capacitance of an uncovered electrode and 0.3 being the normalised capacitance with cells completely covering an electrode. The capacitance, C , is derived simply from the impedance results using $C = 1/(2\pi fZ'')$, as explained in Section 2.5. Transforming the data of Fig. 6 in this manner (Supplementary Information Fig. S3) shows the capacitance for a single IC with Caco-2 cells exhibited a reduction in normalised capacitance from 1.0 to 0.4. Using the linear relationship, this represents electrode coverage

of 86%. It is also noted that the capacitance stabilised at 3 days which is probably indicative of the cells becoming confluent. These results are therefore in broad agreement with the model. Unfortunately, it was not possible to correlate electrical results with images of the electrode array since the epi-illuminated microscope used in this work was incapable of imaging the flattened monolayer (the cells appearing transparent). This ECIS experiment therefore demonstrates the ability of the low-cost post-processed CMOS IC technology to function as an impedance sensor. Reversibility of the impedance changes by cleaning of the arrays indicated that the measured effects were due to cell growth rather than damage to the device, for example corrosion. This is further supported by the fact that controls were run with the media but no cells, so if corrosion was occurring, it would have resulted in Z'' changes in these devices also, but no such effect was observed. Furthermore, re-use of the ICs demonstrates the corrosion resistance of the modified electrodes and the stability of the IC packaging to at least 40 days.

As an economic method of forming generic biocompatible CMOS electrodes, the process is not limited to cell-based biosensors and can be used to form transducers in many other types of electrochemical biosensor. Potential applications therefore include medical diagnosis and monitoring (e.g. wound healing), medical implants (e.g. glucose monitoring), environmental monitoring, detecting pathogens in food and water and for defence through the detection of biological agents (Velusamy et al., 2010).

Further work is in progress to demonstrate that the anodising and post-processing can be performed via on-chip circuits. It is intended that anodisation is performed with zero silicon area overhead by using only the standard electrostatic discharge (ESD) protection circuits that must be present in any commercial (high yield and reliable) CMOS device. Additionally, the authors will assess the technology as a biosensor of harmful biologic agents (e.g. saxitoxin): this will evaluate open porous alumina electrodes and porous alumina filled with gold, but with a smooth surface more suited to sensing antigen binding. With regards to commercialisation, initial products are likely to require the post-processing to be performed after packaging, as in this work. This requires anodisation and plating counter electrode configurations that are specific to the packaging. A preferred approach is therefore to post-process the devices at the wafer-level, prior to application-specific assembly. This would require a low-resolution (millimetre scale) mask, but would still not require complex lithography or a clean-room environment. This approach would, however, require biasing of the wafers during post-processing and therefore requires further research to assess its feasibility. Wafer-level processing would aid suitability of this technology for applications requiring no IC wire-bonds, such as monolithic wireless devices utilising radio frequency identification (RFID) and medical implants.

5. Conclusion

A simple method has been established by which aluminium electrodes realised in standard CMOS technology can be made biocompatible and sense both the fast electrical activity of neurons and the slow changes in impedance of growing and dividing cells. Since the devices are based on ubiquitous CMOS technology the integrated circuits can be readily developed, with minimal additional fabrication costs, to include appropriate amplifiers, filters and wireless subsystems, thus reducing the complexity and cost of the external recording system. By using standard semiconductor fabrication techniques and well-established technologies, such devices can form the basis of a wide range of applications including single-use (disposable) biosensors in high throughput drug discovery assays, neuroprosthetics and as a basic research tool in biosciences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2011.11.015](https://doi.org/10.1016/j.bios.2011.11.015).

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