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Self-Calibrating and Multiplexed Electrochemical Lab on Chip for Cell Culture Analysis and High-Resolution Imaging

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In vitro analysis requires cell proliferation in conditions close to physiological. Lab-on-a-Chip (LoC) devices simplifies, miniaturizes and automates traditional protocols, with the advantage of being cheaper and faster thanks to the shorter diffusion distances. Main limitation of current LoCs is still to control the culture conditions. Most of LoC employ off-chip equipment to determine cell culture activity, which confers a limited monitoring capacity. The few systems integrating transducers on-chip present important functional problems mostly associated to the attachment of biomolecules to the transducer surface (i.e. biofouling) and the impossibility to re-calibrate the sensors during cell culturing. This limitation is settled in the present LoC containing a network of micro-channels and micro-chambers, which allows: (i) cell seeding and cultivation avoiding biofouling risk, (ii) multiplexed analysis of cell culture, reactivation and recalibration of the (bio)sensors without compromising cells viability, (iii) cells imaging and (iv) reference electrode compartmentalization to guarantee its stability. The activity of the culture is monitored with four independent electrochemical micro-electrodes for glucose, hydrogen peroxide, conductivity and oxidation reduction potential. Electrochemical analysis is complemented with high-resolution confocal microscopy analysis. This paper demonstrates the suitability of the current configuration for cell culture monitoring and future application to drug screening or organ-on-a-chip development.

**Introduction**

With important ethical issues arising from animal experimentation, cell cultures in relevant environments are positioning as the best alternative for studying the causes of diseases or drug screening. Conventional in vitro tests are now competing with simpler, miniaturized and automated Lab-on-a-chip (LoC) devices, which minimize human errors and sample contamination, while decreasing the cost and duration of the assays.

Main limitation of current LoCs for cell culture is on cell culture monitoring. Cells proliferating on the chip should be continuously analysed to guarantee that they are growing at optimal physiological conditions. Only if so, the changes reported in the culture may be associated to the process or treatment under study. Unfortunately, most of LoCs do not integrate transduction systems but require external benchtop equipment (i.e. microscope or an imaging system) to record cell activity. From the few systems integrating the transducer on-chip, three have been already commercialized, demonstrating the potential market of such technologies. They are: the DOX-96, commercialized by Dalkin Industries Ltd, which monitors cell respiration; the BIONAS 2500 from the Biosas GmbH company, which monitors extracellular pH, cell adhesion and cellular respiratory activity; and xCELLigence Real-Time Cell Analyser from ACEA Biosciences, which evaluates cell adhesion and cytotoxicity through impedance spectroscopy. It worth mentioning that, from the three technologies only the last one is still commercially available. The reason for that may be partially associated to the transduction mechanism. xCELLigence uses impedance spectroscopy to report on cell attachment, and correlate such measurement with cell migration and viability. Since cell attachment measurements are a particular case of biofouling, this system is not affected by this common drawback. In contrast, DOX-96 and BIONAS 2500 integrate amperometric sensors, which are very sensitive to biofouling. Biofouling reduces the active area of the electrode, altering amperometric recordings. For reliable results, amperometric sensors should be reactivated and recalibrated between measurements. Regarding the latter, it is becoming increasingly accepted that the integration on-chip of electrochemical transducers should consider important design constraints to guarantee cell integrity in physiological conditions and measurement reliability.

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These constrains have not been completely fulfilled by current cell culture systems. A number of examples integrate CO$_2$, glucose and oxygen sensors on poly(dimethylsiloxane) (PDMS) structures. These systems take benefit from the low cost, fabrication simplicity and high gas diffusion capacity of PDMS, although not solving the biofouling problem of electrochemical transducers. Besides, there is some controversy regarding the chemical stability of PDMS since some authors claim that PDMS releases un-polymerized monomers to medium that may affect cell activity. Silicon and glass are more chemically stable but also more expensive and difficult to bond. For this reason, the number of publications using these materials is small. In 2003, a silicon chip with pH-ISFETs and amperometric electrodes was proposed for cell proliferation and analysis. Unfortunately, in the design fluidic inlets and outlets were on top of the silicon chip platform, which did not allow simultaneous imaging of cells. This limitation was solved in 2014 when Urban et al. developed a new LoC device integrating electrochemical sensors with a transparent tape enabling imaging. However, the microfluidic configuration of the chip, with a single micro-channel connecting the fluidic inlet and the outlet, did not allow sensor reactivation and recalibration between measurements, compromising the reliability of the analysis.

As a step forward, we present a silicon-glass electrochemical LoC with compartmentalisation and smart micro-fluidic interconnections enabling: (i) cell seeding and proliferation in an independent micro-chamber minimizing biofouling; (ii) regular reactivation and recalibration of the amperometric electrodes without compromising cell viability; (iii) compartmentalisation of the Ag/AgCl pseudo-reference electrode to enhance stability; (iv) culture medium and reagents transport with small dead volumes; and (v) high-resolution imaging of cells through a micro-patterned glass layer. Integration of four electrodes allows multiplexed analysis of key analytes, namely glucose, hydrogen peroxide, conductivity and oxidation reduction potential (ORP). Micro-electrodes have been tested individually and all together to evaluate their performance. Human lung fibroblasts cells (MRC-5) have been cultured in the multiplexed LoC (ME-LoC) and oxidative stress induction by hydrogen peroxide has been evaluated with the four micro-electrodes integrated and compared to optical analysis by confocal microscopy.

Experimental

Design and Fabrication of the ME-LoC

The ME-LoC is composed of two layers permanently attached by anodic bonding. The bottom silicon layer contains the electrochemical transducers, connections and pads. Microfluidic elements, i.e. micro-channels and micro-chambers, are defined in the second layer, a glass cover, which seals the system. A schematic image of the ME-LoC is shown in Fig. 1A. The ME-LoC contains three independent, but fluidically connected, micro-chambers: (i) a 15-µL cell culture chamber for cell seeding and proliferation; (ii) a 15-µL measurement chamber integrating four electrode systems for amperometric analysis of cells; and (iii) a 6-µL pseudo-reference chamber containing the reference electrode for amperometric measurements. A network of fluidic micro-channels is designed, with multiple inlets and outlets, for a precise microfluidic control while minimizing dead volumes. With this network it is possible to seed cells without affecting amperometric measurements, to rereactivate/recalibrate the electrodes in situ without altering cell proliferation and to set the reference electrode conditions without influencing the measurement or cells.

Five thin-film gold electrode systems are defined in the silicon chip, one in the pseudo-reference chamber and four in the measurement chamber. The pseudo-reference chamber...
contains a circular gold electrode of 3.1 mm² to operate as pseudo-reference electrode in electrochemical and ORP measurements (after electrodeposition of Ag/AgCl layers). In the measurement chamber, systems (1), (2) and (3) in Fig. 1A consist of two circular gold electrodes of 0.6 mm² and 1.2 mm² to be used as working and counter electrodes, respectively, in amperometric measurements. The electrochemical system (4) contains 4-bars gold electrodes for conductivity measurements. Both silicon chip and glass cover were fabricated using standard silicon technology, as summarized in Fig. 1B. First, (step 1) the silicon wafer was etched in the regions of the pads, electrodes and connections and subsequently passivated by deposition of a thermal silicon oxide layer for protection. Additional silicon nitride and aluminium layers were deposited on the rear side of the wafer as passivation for subsequent etching steps. Next, (step 2) rear fluidic connections were developed in a multi-step process involving two photolithographic and three etching steps (two deep reactive ion etching, DRIE, and one RIE). Before completing the opening of the rear fluidic connection (step 3), gold electrodes, electric connection and pads were defined through a lift-off process. Rear fluidic connections were finally defined by RIE. Final step (step 4) consisted of the anodic bonding between the silicon chip and the glass cover, which previously patterned by etching. Anodic bonding was performed at 1000 V and 400ºC for 30 min at wafer level with the SBG Gen2 SUSS MicroTec (SUSS MicroTec Group, Garching, Germany) (Fig. S1A, in the ESI). The thickness of the glass cover (about 180 µm) was chosen to enable high-resolution imaging. Afterwards, the glass-silicon wafer was diced into individual chips (Fig. S1B, in the ESI) and fluidic tubes were inserted to the rear fluidic connections of the chip with epoxy resist (Fig. S2A, in the ESI). Then, chips were bonded to a printed circuit board (PCB), electrically connected by wire bonding (Fig. 1C) and protected with a thin isolating silicone layer to preserve the electrical connections (Fig. S2C and D in the ESI). The rear fluidic connections enabled fluid management and electrochemical measurements without interfering imaging. The final assembled ME-LoC system is illustrated in Fig. 1D.

Reagents

All reagents were of analytical grade, or equivalent, and were purchased from Sigma Aldrich, unless otherwise stated. All solutions were prepared using de-ionized water.

Equipment

For the electrodeposition of the polypyrrole (PPy) layers used to entrap enzyme and mediators, an Autolab electrochemical workstation (PGSTAT-100 potentiostat - galvanostat, Ecochemie, Uthecht, The Netherlands) controlled through NOVA software was used. Electrodeposition required an external reference (Orion 92-02-00, Thermo Fisher Scientific Inc., Beverly USA) and counter electrode (Platinum layer, Radiometer, Lyon, France). To ensure electrical contact between on-chip and off-chip electrodes, all of them were immersed in the same 0.1 M KCl solution during electro-synthesis.

Multiplexed electrochemical analysis on-chip was performed with a portable homemade multi-parametric system fabricated at the IMB-CNM. Briefly, the system generated and received signals from four micro-sensors: (i) the resistance temperature detector (RTD) Pt-100 (Pico Technology, St Neots, UK); (ii) the four-wires conductivity sensor, where an alternating current at 5 kHz is applied to the two external electrodes and the current is recorded between the two internal electrodes; (iii) the ORP sensor, directly connected to the acquisition module and providing potential values by comparison with the reference electrode; and (iv) the amperometric sensors, a three-electrode microsensor system where the electrical voltage was fixed between the reference and the working electrodes while recording the current flowing between the working and the counter electrodes. The device incorporated two amperometric terminals that could be used simultaneously for glucose and hydrogen peroxide determination. The digital interface permitted to stabilize the communication between the user and the analogue electronic part. The visualization of the results and the configuration of the measurement parameters were carried out employing a virtual instrument programmed with LabView 2013 (National Instruments, Austin, USA).

Fluorescence microscopy images were acquired with a Nikon Eclipse Ti-E inverted microscope (Plan Apo 60x Oil DIC objective; Nikon Instruments, Tokyo, Japan) using the same imaging settings in either non-treated or H2O2-treated conditions. Dihydroethidium (DHE) fluorescence was detected with a 604/40 nm band-pass filter. For quantification, integrated optical density was calculated in each cell (79 ± 16 cells by experimental condition) using NIS Elements AR 4.13 software (Nikon Instruments, Tokyo, Japan).

Ag/AgCl pseudo-Reference Electrode

The electro-generation of the Ag/AgCl pseudo-reference electrode is based on the protocol described in. First, the thickness of the Ag and AgCl layers was estimated by the combined Faraday law:

\[
\text{th} = \left( \frac{I \times t \times M_W}{F \times p \times A} \right)
\]

where th is the thickness of the formed layer (in cm), I is the current intensity (in A), t is the electrodeposition time (in s), M_W is the molecular weight of the electrodeposited specie (107.87 g/mol for silver and 143.32 g/mol for silver chloride), F is the Faraday constant (96485.33 C/mol), p is the density of the electrodeposited species (10.5 g/cm³ for silver and 5.56 g/cm³ for silver chloride) and A is the area of the electrode (0.031 cm²). Based on theoretical data, a Ag layer was galvanostatically electrodeposited on the working electrode. For a 4 µm layer, Ag was electrodeposited for 3760 s at -31 µA in a 10 mM HNO₃ solution containing 15 mM tartaric acid and 0.1 M silver nitrate (AgNO₃). The Ag layer was then partially chlorinated to obtain a 1.3 µm-thick layer in a 0.1 M HCl solution containing 0.1 M potassium chloride (KCl). Chlorination conditions were +12 µA for 1250 s. The thickness of the layers was confirmed by profilometry (Figure S3, in the ESI).
Electrochemical Measurements

Four parameters were independently determined through electrochemical transduction, namely hydrogen peroxide, glucose, conductivity and ORP. Each (bio)sensor was characterized first individually and later in combination with the others. Sensors characterization and optimization was conducted on-chip using PB (pH 5.5) for being the optimal conditions for glucose oxidase activity. Cell culture analysis was performed in culture medium (pH 7). Before analysing cell proliferation, the response of the glucose biosensor in the culture medium was determined to evaluate matrix effects and the influence of the pH in the enzymatic activity. In the characterization, all samples were supplemented with suitable concentrations of the analyte. Sensitivity, limit of detection and lineal range were determined. Each parameter was measured in triplicate.

Glucose Biosensor

The glucose enzymatic biosensor was produced in the electrode system (2) (Fig.1A) by selective electrodeposition of PPy \( \text{PPy}^{27,28} \) layers on the working electrode surface. Two PPy layers were consecutively electrodeposited, the first one containing the mediator (ferricyanide) and the second one the enzyme (glucose oxidase, GOX; GOX activity described in Fig. S4A, in the ESI). The enzymatically-mediated production of ferrocyanide was proportional to glucose concentration and was determined amperometrically at + 0.15 V (vs. Ag/AgCl).

The electrodeposition solution contained 0.4 M pyrrole (monomer), 0.1M KCl (counter-ion) in phosphate buffer (PB = 0.05 M KH\(_2\)PO\(_4\), pH 7) and either 100 mM of potassium ferricyanide (mediator) for the mediator layer or 5 mg/mL GOX for the enzymatic layer. PPy layers were electrodeposited at +0.7 V (vs Ag/AgCl pseudo-reference) until reaching an accumulation charge of 250 mC cm\(^{-2}\) (approx. 50 s, Fig. S5B in the ESI) for the mediator layer or 500 mC cm\(^{-2}\) (approx. 50 s, Fig. S5B in the ESI) for the enzymatic layer. Electrodeposition was performed using the electrodes integrated on the chip. The glucose biosensor was finally rinsed with buffer and stored in PB solution at 4ºC until used.

Linear sweep potentiometry from 0.8 to 0.0 V (vs Ag/AgCl pseudo-reference) at scan rate of 25 mV s\(^{-1}\) was used to determine glucose concentration through the oxidation of the enzymatically-reduced ferrocyanide. Glucose concentrations between 0 and 50 mM in PB were analysed. Matrix effects were determined by comparison of previous PB records with those obtained from culture medium samples spiked with glucose (between 0 and 1 mM glucose).

Hydrogen Peroxide Sensor. Hydrogen peroxide was determined amperometrically with the electrode system (1) (Fig.1A). Chronoamperometric measurements were carried out at +0.7 V (vs Ag/AgCl pseudo-reference) for 3 min in PB solutions supplemented with hydrogen peroxide (from 0 to 3 mM). The recorded average faradaic current was directly proportional to the concentration of hydrogen peroxide in the sample.

ORP Sensor. The ORP is the potential generated from the equilibrium between the electron activity of the sample, and the adsorption/desorption processes at the electrode surface according to the Nernst equation. Due to the nonspecific nature of this measurement, i.e. it refers to the global electron activity of the medium, its application in biotechnology is limited. However, it is now quite accepted that ORP can be a valuable indicator of metabolic activity in fermentation processes and cell proliferation. ORP measurements were performed with the electrode system (3) in Fig.1A. The working Au electrode and the integrated Ag/AgCl pseudo reference electrode were used to set the redox potential of the solution. The final potential was collected after 3 min recording. Calibration was carried out with two calibration solutions of 220 mV and 468 mV (Sigma-Aldrich).

Conductivity Sensor. The 4-bars electrode system (electrode system (4) in Fig.1A) was used to determine conductivity in PB solutions from 0.80 to 13.07 mS cm\(^{-1}\). In conductivity measurements, an alternating current of 8 mV at a fixed frequency of 5 kHz (corresponding to the frequency value associated to solution resistance) was applied between the electrodes located more externally, and signal recordings (in mV) were acquired with the two internal electrodes.

Multi-parametric Measurements. Joint measurement of all parameters was conducted to determine cross-talk between transduction methods and interference of one analyte in the determination of another. Three sets of samples were prepared, changing either glucose (from 0 to 10 mM), hydrogen peroxide (from 0 to 1 mM) or conductivity (from 100 to 700 Q cm), while maintaining constant the concentration of the others components. Each sample in the set was sequentially measured with the four electrochemical (bio)sensors integrated on-chip in triplicate.

Cell Culture

Human fibroblast cell line MRC-5 from the ATCC Line Bank (Virginia, USA) was used to validate the ME-LoC. MRC-5 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 25 mM glucose concentration and supplemented with 10 % foetal bovine serum and 1% antibiotic solution (100 kU/mL penicillin and 10 mg/mL streptomycin) in a humidified incubator with 5% CO\(_2\) at 37ºC. When confluent, MRC-5 cells were passaged with 0.25% trypsin-EDTA (Gibco, Fisher Scientific, Spain) and an aliquot was stained with trypan blue for cell counting. Then, cells were centrifuged and resuspended in complete DMEM at a concentration of 10^6 cell/mL and seed either in the ME-LoC or in conventional plastic plates for comparison. Proliferation and oxidative stress induction experiments were conducted with DMEM supplemented with 2
Oxidative Stress Induction and Detection

Oxidative stress in MRC-5 cultured cells was induced by addition of H₂O₂ according to previous protocols [30,31]. Cells proliferating in conventional 12 mm glass culture coverslips were exposed to H₂O₂ for 30 min in the humidified cell incubator (H₂O₂ concentration: 10, 15, 25 and 50 µM). Intracellular reactive oxygen species (ROS) levels, including superoxide anion, were determined by means of the oxidation-sensitive fluorescent probe dye DHE (Ex/Em=535/610 nm). The detection protocol was the following. After oxidative stress induction, cells were washed with phosphate buffered saline (PBS) and incubated in 2 µM DHE at 37°C for 30 min [31,32]. Oxidative stress induction data represent the means of at least two independent experiments (means ± SEM). The data were analysed using GraphPad Prism 5 software (San Diego, CA, USA). The Student’s t-test or one-way analysis of variance with post hoc analysis using Bonferroni’s multiple comparison test was used for parametric data. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Characterization of Electrochemical (bio)Sensors, Culturing and Imaging in the LoC

Ag/AgCl pseudo-Reference Electrode Stability. The stability of the integrated Ag/AgCl pseudo-reference electrode was evaluated by monitoring the potential drift over time in 1 M KCl at Open Circuit Potential (OCP). Experimentally, the resting potential between the reference electrode under test and a commercial double junction Ag/AgCl (3.0 M KCl) reference electrode, considered as standard electrode, was determined over time. Both the ME-LoC and the external reference electrode were immersed in the same 1 M KCl solution to ensure electrical contact. Short term (12 h) and long term (5 days) experiments were conducted. Results from the integrated Ag/AgCl pseudo-reference electrode were compared with those obtained with an integrated Au pseudo-reference and a commercial double junction Ag/AgCl (3.0 M KCl). After 12 h of analysis (Fig. S6, in the ESI), the potential drift corresponding to the integrated Ag/AgCl pseudo-reference electrode was 11 mV, 5 times higher than commercial double-junction reference electrodes (2 mV) but 5 times lower than integrated Au pseudo-reference (55 mV). Similarly, the long-term stability for 5 days of the integrated reference electrode was always better than the Au pseudo-reference but worse than the commercial reference electrode. Even though, the potential variation over time was small enough to guarantee stability, accuracy and reproducibility of the amperometric, conductimetric and ORP measurements. Additionally, compartmentalization contributed to avoid one of the main problems of the reference electrodes which is the direct contact of the reference with the test and sample solutions never reached the pseudo-reference chamber, which was always filled with 1M KCl.

The position of the pseudo-reference electrode in an independent chamber and at a distance of the working and counter electrodes may negatively influence amperometric measurements. To evaluate this, the response of the integrated electrodes (working, counter and reference electrodes in the ME-LoC) to ferricyanide was compared with an external double-junction reference electrode (working and counter electrodes of the ME-LoC). The study was complemented with a third experiment with external electrodes operating as working, counter and reference electrode. Although there was a small shift in the potential peak, the three situations provided comparable results (Fig. S7 in ESI) with similar current values. No potential drop associated to hysteresis was observed in the case of integrated electrodes probably due to the good electrical conductivity through the fluidic micro-channels.

Electrochemical Measurement of Individual (bio)Sensors on-Chip. Each (bio)sensor in the ME-LoC was characterized individually. First, microelectrodes in the ME-LoC were electrochemically activated by cyclic voltammetry in 0.1 M KNO₃ (20 scans; potential range = +0.8 V to -2.2 V vs. Ag/AgCl pseudo-ref; scan rate = 100 mV s⁻¹) [34]. After that, a cyclic

![Fig. 2. Calibration of the electrochemical (bio) sensors. (A) Calibration curve for the hydrogen peroxide sensor using the mean current value of the last 30s of the recorded signal for a concentration of hydrogen peroxide of 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2, 3 and 5 mM; (B) Least squares analysis of ORP for the standard solutions of 220 mV and 468 mV. The dotted line corresponds to ideal correlation between real (measured) and theoretical values; (C) Calibration plot for the 4-bars sensor using conductivity PB solutions of 0.80, 1.52, 2.82, 6.87 and 13.07 mS cm⁻¹; (D) Calibration curve of the PPy/ferricyanide/GOX glucose biosensor in PB solution (black line) and in the cell culture medium (red line). Each point represents the current value recorded from the sweep line potential obtained from a glucose concentration of 0.1, 0.3, 0.6, 1, 2, 3 and 5 mM. Error bars represent the corresponding standard deviation from two chips measured in triplicate (n=3).](image-url)
voltagemogram of ferricyanide was acquired and used to evaluate the active area of the working electrode. The activation process was repeated until obtaining repetitive ferricyanide voltammograms\textsuperscript{34}. Results from this characterization are presented in Fig. 2.

Hydrogen peroxide (Fig. 2A) was determined by chronoamperometry at +0.7 V (vs Ag/AgCl pseudo-reference). The average current density increased with the hydrogen peroxide concentration in the range from 0.001 to 1 mM with good sensitivity (slope = 1.8 ± 0.2 µA mM\textsuperscript{-1}) and linearity (r = 0.998). The calculated limit of detection (LOD) was 5×10\textsuperscript{-4} mM, according to the 3σ IUPAC criteria\textsuperscript{31}.

Calibration of ORP sensor was performed by measuring two calibration solutions of known potential, i.e. 220 and 468 mV according to the supplier. Following conventional calibration protocols for ORP, the theoretical values were compared to the ones obtained experimentally (214.3 ± 0.5 mV and 470.4 ± 1.0), which presented a variation coefficient below 3% in any case. A paired representation of theoretical versus experimental data (Fig. 2B) resulted in a straight line with a slope of 1 (1.0± 0.1), confirming the good performance of ORP sensor. The conductivity sensor was characterized with KCl solutions (Fig. 2C), providing results comparable to a commercial conductimeter. The ME-LoC presented good sensitivity (0.14 ± 0.01 mV Ω\textsuperscript{-1} cm\textsuperscript{-1}) and linearity (r\textsuperscript{2} = 0.997) in the range between 0.80 and 13.07 mS cm\textsuperscript{-1} (Fig. 2C), providing results comparable to a commercial conductimeter.

For the glucose biosensor, the current magnitude at +0.15 V (vs Ag/AgCl pseudo-reference) corresponding to the oxidation of the enzymatically-produced ferrocyanide was used as an analytical signal. The linear range of the biosensor comprised three orders of magnitude, from 0.1 to 25 mM, with good sensitivity (0.0060 ± 0.0003 µA mM\textsuperscript{-1}) and linearity (r = 0.998) (Fig. 2D). Matrix effects were investigated with the glucose biosensor for being one of the most sensitive to biofouling and pH. Results are presented in Fig. 2D (red dots/line). As shown, similar responses were obtained in both buffer and culture medium solutions, providing comparable sensitivities (0.052 ± 0.003 and 0.041 ± 0.001 µA mM\textsuperscript{-1} for PB solutions and cell culture medium, respectively) and linear ranges. However, a small displacement of the calibration curve to higher current magnitudes was observed with the culture medium due to matrix effects. These effects were considered in subsequent measurements.

It is worth mentioning that the linear range of the sensor was in agreement with the expected magnitudes of each parameter: between 0 to 2 mM for glucose (considering that the culture medium was supplemented with 2 mM glucose and that this parameter should decrease during the experiment due to cell metabolism); from 100 to 250 mV for ORP considering the composition of the culture medium; around 25 mS cm\textsuperscript{-1} for conductivity, which may slightly increase or decrease along the experiment; and from 1 to 50 mM in the case of hydrogen peroxide, which was the range conventionally used to induce oxidative stress in mammalian cells.

Fig. 3. Multiplexed electrochemical analysis and cell culture imaging. Cross-talk results changing the glucose (A) in a concentration range from 0 to 2 mM, the hydrogen peroxide (B) concentration in a range from 0 to 1 mM and (C) the conductivity in a range from 0.8 to 13.07 mS cm\textsuperscript{-1}. (D) Cell culture microscopy image. Error bars represent the corresponding standard deviation from two chips measured in triplicate (n=3) in Fig. 3.

Glucose (Fig. 3A) and hydrogen peroxide concentrations (Fig. 3B) were selectively recorded by the corresponding (bio)sensors and did not interfere the other measurements. Thus, there was not cross-talk in the determination of these two parameters. Although changes on medium conductivity have been reported to influence ORP\textsuperscript{35}, only minor variation (around 30 mV) was
observed in this case (Fig. 3C) as expected from the small influence of conductivity to the Nernst equation. Thus, conductivity, glucose or hydrogen peroxide concentrations were not expected to interfere the determination of any other of the analytes of interest in these assays. Additionally, any influence of conductivity was minimized by using culture media with high and constant conductivity.

**On-Chip Culturing and High-Resolution Imaging.** To validate cell growth and imaging on-chip, the culturing micro-chamber was selectively filled with MRC-5 cells. When confluent, cells were stained with DAPI (nucleus) and eosin (cytoplasm) for high-resolution imaging. Microscopy images were acquired at 20x showing the characteristic adherent fibroblast-like cell phenotype of this cell type (Fig. 3D). These results validate the glass-silicon ME-LoC for cell culturing and high-resolution imaging.

**Fig. 4. Fluidic control of the ME-LoC.** (1) Filling of the pseudo-reference electrode with a blue-coloured solution; (2) Filling of the cell culture chamber with a red-coloured solution; (3) Initial calibration of the electrochemical (bio)sensors using a purple-coloured solution; (4) Medium culture measurement by the electrochemical (bio)sensors in the measurement chamber; (5) Final calibration of the electrochemical (bio)sensors.

**Fig. 5. Induction of reactive oxygen species (ROS) in MRC-5 cells.** Top images correspond to optical (right), fluorescence (left) and mergence (middle) images of MRC-5 cells (non-treated cells, NT) after staining with the membrane-permeable ROS-sensitive dye dihydroethidium (DHE). Below, fluorescent images of MRC-5 cells stained with DHE after 30 minutes of incubation with the hydrogen peroxide (H2O2) indicated in the image. The plot in the bottom represents the variation of the relative intensity of DHE-dye, from image quantification, with the H2O2 concentration. ** P<0.01; *** P<0.001 compared to NT cells. Error bars represent the corresponding standard deviation. Three technical (n=3) and biological (N=3) replicates were conducted.
Microfluidic Control of the ME-LoC. The selective filling/emptying of the micro-chambers and the diffusion of molecules to neighbouring reservoirs was evaluated with high contrast colorants. Different colorants were injected to each micro-chamber following a defined protocol and specific inlets/outlets to demonstrate that the ME-LoC was able to: (i) create a stable and controlled environment for the reference electrode, (ii) seed and proliferate cells in an independent micro-chamber, (iii) reactivate/recalibrate the electrodes without interfering cell proliferation, (iv) monitor cell activity (Fig. 4). In all cases a flow rate of 3 µl s⁻¹ was applied between the fluidic inlet and outlet in use. Other fluidic inlets and outlets remained closed to minimize liquid flowing to other regions of the chip.

To demonstrate (i), the pseudo-reference micro-chamber was filled with a blue colorant using the fluidic connection number 1 as inlet and the number 2 as outlet (Fig. 4.1). As shown in the magnification of the figure, the blue colorant was confined in the micro-chamber with minimal diffusion to other chambers due to the small diameter of the connecting micro-channels. Based on that, the pseudo-reference electrode micro-chamber could be filled with a 0.1 M KCl solution to ensure the stability of the reference electrode potential without compromising electrochemical measurements or cell viability. In a second step (Fig. 4.2), the culture micro-chamber was filled with a red colorant to demonstrate (ii). Selective filling was achieved by using the connection number 4 as inlet and the number 5 as outlet. The minimal diffusion of the red colorant (see the magnification of the image) guaranteed the confinement of cells in the cell culture micro-chamber, minimizing them to flow to other regions of the chip. This fact minimized the biofouling risk during cell culture. The demonstration of (iii) required the selective filling of the measurement chamber. In Fig. 4.3, the measurement chamber was filled with a purple solution. The fluidic connection number 3 was used as inlet and the number 6 as outlet. As shown, there was minimal diffusion to other chambers (see image magnification), which demonstrated that it was possible to reactivate and recalibrate the electrodes before measurements, without compromising cell proliferation. Finally, to prove (iv), the culture medium was selectively transferred from the culture micro-chamber to the measurement micro-chamber for measurement (Fig. 4.4). The red colored culture medium was thus transported from one chamber to the other by using the fluidic connection number 5 as inlet and number 6 as fluidic outlet. As an additional step, the measurement micro-chamber was again filled with the purple solution for reactivation and recalibration of electrodes before a new measurement was performed (Fig. 4.5). In this case, fluidic connection number 3 and number 6 were used again as inlet and outlet. A video recording all these steps in real time is included in the ESI of this article.

Results demonstrated the compartmentalization, minimal diffusion and versatility of the intricate network of micro-channels designed in the chip, proving high control of cell culture for the integration of multiple micro-electrode systems in the chip and their self-calibration capacity.

Off-Chip Optimization of Oxidative Stress Induction. Oxidative stress is involved in many pathologies, such as cancer, Alzheimer and cardiovascular disease. For this reason, a well-known oxidative stress induction model using hydrogen peroxide as inducer was selected for the validation of the ME-LoC. Prior to on-chip analysis, oxidative stress induction was optimized off-chip. As shown in Fig. 5, incubation of the cells with increasing concentrations of H₂O₂ revealed a significant induction of intracellular ROS levels, reported as an increasing level of DHE intensity. Maximal induction of the tested dose was obtained between 25 and 50 µM. Above these values (100 µM of H₂O₂), cells showed an important morphological alterations affecting viability (data not shown). These levels of ROS induction were similar to those observed in other adherent fibroblast-like cells exposed to H₂O₂. The range from 0 to 50 µM H₂O₂ was chosen for ME-LoC validation. It is important to remark that this hydrogen peroxide concentration range was in agreement with the linear range of the hydrogen peroxide sensor integrated in the ME-LoC system, which was between 1 and 1000 µM H₂O₂.

On-Chip Multiplexed Analysis of Oxidative Stress Induction in the ME-LoC. The performance of the ME-LoC was evaluated using the oxidative stress induction protocol optimized before. Briefly, MRC-5 cells suspended in DMEM supplemented with 2mM glucose were seeded in the culture chamber (Fig. 6A). The ME-LoC was then introduced in the incubator for cell proliferation at optimal conditions (5% CO₂ and 37°C). After 24 h of incubation, micro-electrodes in the ME-LOC were reactivated and a cyclic voltammogram of ferricyanide was acquired and used to evaluate the activation process (Fig. 6B). Micro-electrodes were then recalibrated at a single point with calibration solutions of glucose (1 mM glucose) hydrogen peroxide (0.1 mM H₂O₂), conductivity (2.82 mS cm⁻¹) and potential (220 mV). Recalibration results are shown in Fig. 6C. As previously stated, the calibration process did not affect cell viability thanks to the compartmentalization of the system. Oxidative stress was induced by incubation of the cells to hydrogen peroxide. After 30 min of incubation with the reagent, culture medium was driven to the measurement micro-chamber and analysed using the four electrode systems implemented in the ME-LoC. Glucose, hydrogen peroxide, conductivity and ORP were thus determined.

The study was complemented with confocal microscopy imaging and analysis of oxidative stress induction using the fluorescent indicator DHE. To this end, the DHE solution was inoculated to the cell culturing micro-chamber, incubated for 30 min and imaged by confocal microscopy.
After analysis at one hydrogen peroxide concentration, the ME-LoC was regenerated for future experiments as follows. Cells were detached with 0.025% trypsin (5 min). The micro-chamber was then washed with 70% ethanol (3 times) and PBS (5 min, 3 times), removing any remaining present in the chip and leaving it ready for next assays. The performance of the electrodes was also evaluated at this point the glucose biosensor was regenerated by electrodeposition with PPy, as described in the experimental section.

The oxidative stress induction procedure was repeated by the following concentrations of hydrogen peroxide: 10, 15, 25 and 50 µM. Electrochemical analysis of cell culture provided the following results, illustrated in Fig. 6D. Hydrogen peroxide sensor reported an increase in the response (blue coloured line, blank circles) when increasing the concentration of this molecule in the culture medium. Considering calibration, the reported hydrogen peroxide concentration was very close to the inoculated concentration. The glucose biosensor signal (blue coloured line, filled blue coloured circles) increased with the hydrogen peroxide concentration up to 25 µM H₂O₂, where it stabilized. This increase in glucose concentration in the medium may be attributed to a reduction of glucose consumption due to oxidative stress and cell death. At 15 µM, the magnitude of the current recorded corresponded to a glucose concentration of 2 mM, which suggested that at this hydrogen peroxide concentration oxidative stress induction killed almost all the cells in the ME-LoC. The signal corresponding to the conductivity sensor (red coloured line, blank circles) was almost constant over time, providing a constant potential of 24 ± 2 mS cm⁻¹. This magnitude coincided with the medium conductivity and thus, conductivity changes associated to breaking of the cell membrane and the massive release of ions could not be recorded with the current configuration of the ME-LoC. On the other hand, the ORP signal (red coloured line, filled red coloured circles) increased with the increasing hydrogen peroxide concentration up to 25 mM. This increase in the ORP magnitude may be associated to a reduction of metabolites production, as suggested in the literature, or the increase of hydrogen peroxide concentration.

Electrochemical results were complemented with high-resolution confocal imaging of the oxidative stress indicator DHE (black coloured line, filled black coloured circles; image of stained cells in Fig. 6E). An increase of oxidative stress was
recorded up to 25 µM hydrogen peroxide, coinciding with electrochemical data. This result confirmed the good performance of the ME-LoC in the analysis of oxidative stress induced by hydrogen peroxide, as an example of process with high impact in pathology development.

Conclusions
The results presented in this article demonstrate that a precise control of cell culture is possible with a self-calibrating, reusable and multiplexed LoC. The ME-LoC presents a complex network of micro-channels and micro-chambers that allow compartmentalization of the reference electrode; cell seeding and proliferation without biofouling; electrodes reactivation and recalibration; and multiple analyte detection, namely glucose and hydrogen peroxide concentrations, conductivity and ORP, as a way to monitor cell metabolism. Electrochemical analysis is completed with high-resolution imaging by confocal microscopy after labelling with fluorescent dyes. The evaluation of the device with MRC-5 cells by induction of oxidative stress with hydrogen peroxide demonstrates a dose-dependent reduction of glucose consumption and a proportional increase of DHE emission. ORP is also affected by the reduction in cell metabolism but changes in conductivity due to cell lysis are not observed. These result confirmed the good performance of the system in the determination of oxidative stress, a process observed in the initial stages of pathologies such as cancer or cardiovascular diseases. For simplicity, integration, automation, compartmentalisation and micro-fluidic control, we envisage the current technology as a promising alternative for in vitro testing and organ-on-a-chip development in the near future.

Conflicts of interest
There are no conflicts to declare.

Authors contribution
P. G.-G. and J.M. R. carried out the characterization and optimization of the integrated biosensors, as well as the final measurements with cell cultures proliferating in the ME-LoC device. E. G., J.A. P. and C. J.-J. designed and manufactured the device under cleanroom conditions. M. G.-C contribute to device. E. G., J.A. P. and C. J.-J. designed and manufactured the measurements with cell cultures proliferating in the ME-LoC compartmentalization of the reference electrode; cell seeding and proliferation without biofouling; electrodes reactivation and recalibration; and multiple analyte detection, namely glucose and hydrogen peroxide concentrations, conductivity and ORP, as a way to monitor cell metabolism. Electrochemical analysis is completed with high-resolution imaging by confocal microscopy after labelling with fluorescent dyes. The evaluation of the device with MRC-5 cells by induction of oxidative stress with hydrogen peroxide demonstrates a dose-dependent reduction of glucose consumption and a proportional increase of DHE emission. ORP is also affected by the reduction in cell metabolism but changes in conductivity due to cell lysis are not observed. These result confirmed the good performance of the system in the determination of oxidative stress, a process observed in the initial stages of pathologies such as cancer or cardiovascular diseases. For simplicity, integration, automation, compartmentalisation and micro-fluidic control, we envisage the current technology as a promising alternative for in vitro testing and organ-on-a-chip development in the near future.

Notes and references
† Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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Highly compartmentalized Lab-on-a-chip for multi-parametric cell culture analysis and drug screening