

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AXION BIOSYSTEMS, INC.
Petitioner

v.

AGILENT TECHNOLOGIES, INC.
Patent Owner

U.S. Patent No. 11,906,508

**DECLARATION OF CODY A. DANIELS
UNDER 28 U.S.C. § 1746**

I, Cody A. Daniels, hereby declare as follows:

1. I am making this declaration on behalf of Axion Biosystems, Inc. (“Axion”) in the matter of the *inter partes* review of U.S. Patent No. 11,906,508 (“the ’508 patent IPR”). Unless otherwise stated, the facts stated in this declaration are based on my personal knowledge.
2. I am a Research Librarian at the law firm of Jones Day. I received a bachelor’s degree in Linguistics from the University of Texas in Austin, and an MLIS from the University of North Texas. I have worked as an archivist at the Denton County Office of History and Culture, as a metadata technician at Denton County Records Management, and as a records assistant for Jones Day. I am familiar with the operations and procedures of libraries.
3. I make this Declaration based upon my personal knowledge, and experience.
4. Exhibit 1008 is “Real-Time Monitoring of Morphological Changes in Living Cells by Electronic Cell Sensor Arrays: An Approach To Study G Protein-Coupled Receptors,” by Naichen Yu et al., *Analytical Chemistry*, Vol. 78, No. 1, 35-43 (January 2006) (“Yu”).
5. Appendix A to this declaration contains a copy of the Yu paper acquired from the Linda Hall Library. I compared Exhibit 1008 with Appendix A and find Exhibit 1008 to be a true and accurate copy of the Yu paper. On page 10 of Appendix A, there is a date stamp with the date the issue was received. The stamp

states January 11, 2006. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 2006. Page 12 of Appendix A contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as July 27, 1947. Upon entry of that record into the catalog and subsequent processing of the issue containing the Yu article therein, this article would be locatable by keyword search. The 1947 MARC record indicates that the Linda Hall Library has been collecting issues of Analytical Chemistry since 1947. Thus, the library has been adding subsequent issues of Analytical Chemistry to its collection since 1947, including the Yu paper included in Volume 78 published in 2006. All of this indicates that the Yu paper was indexed and publicly available as of January 2006.

6. Exhibit 1017 is “Monitoring electroporabilization in the plasma membrane of adherent mammalian cells,” by Paramita M. Ghosh et al., Biophysical Journal, Vol. 64(5), 1602-1609 (May 1993) (“Ghosh”).

7. Appendix B to this declaration contains a copy of the Ghosh paper acquired from the Linda Hall Library. I compared Exhibit 1017 with Appendix B and find Exhibit 1017 to be a true and accurate copy of the Ghosh paper. On page 7 of Appendix B, there is a date stamp with the date the issue was received. The stamp

states May 27, 1993. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 1993. Page 9 of Appendix B contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as June 13, 1960. Upon entry of that record into the catalog and subsequent processing of the issue containing the Ghosh article therein, this article would be locatable by keyword search. The 1960 MARC record indicates that the Linda Hall Library has been collecting issues of Biophysical Journal since 1960. Thus, the library has been adding subsequent issues of Biophysical Journal to its collection since 1960, including the Ghosh paper included in Volume 64 published in 1993. All of this indicates that the Ghosh paper was indexed and publicly available as of May-June 1993.

8. Exhibit 1018 is “Performance of a thin film microelectrode array for monitoring electrogenic cells in vitro,” by A. Mohr et al., *Sensors and Actuators*, B 34, 265-269 (1996) (“Mohr”).

9. Appendix C to this declaration contains a copy of the Mohr paper acquired from the Linda Hall Library. I compared Exhibit 1018 with Appendix C and find Exhibit 1018 to be a true and accurate copy of the Mohr paper. On page 6 of Appendix C, there is a date stamp with the date the issue was received. The stamp

states December 30, 1996. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 1996. Page 7 of Appendix C contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as March 22, 1990. Upon entry of that record into the catalog and subsequent processing of the issue containing the Mohr article therein, this article would be locatable by keyword search. The 1990 MARC record indicates that the Linda Hall Library has been collecting issues of Sensors and Actuators B since 1990. Thus, the library has been adding subsequent issues of Sensors and Actuators B to its collection since 1990, including the Mohr paper included in Volume 34 published in 1996. All of this indicates that the Mohr paper was indexed and publicly available as of December 1996-January 1997.

10. Exhibit 1019 is “Portable cell-based biosensor system for toxin detection,” by Joseph J. Pancrazio et al., Sensors and Actuators, B 53, 179-185 (1998) (“Pancrazio”).

11. Appendix D to this declaration contains a copy of the Pancrazio paper acquired from the Linda Hall Library. I compared Exhibit 1019 with Appendix D and find Exhibit 1019 to be a true and accurate copy of the Pancrazio paper. On page 8 of Appendix D, there is a date stamp with the date the issue was received.

The stamp states April 28, 1999. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 1999. Page 9 of Appendix D contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as March 22, 1990. Upon entry of that record into the catalog and subsequent processing of the issue containing the Pancrazio article therein, this article would be locatable by keyword search. The 1990 MARC record indicates that the Linda Hall Library has been collecting issues of Sensors and Actuators B since 1990. Thus, the library has been adding subsequent issues of Sensors and Actuators B to its collection since 1990, including the Pancrazio paper included in Volume 53 published in 1999. All of this indicates that the Pancrazio paper was indexed and publicly available as of April-May 1999.

12. Exhibit 1021 is “CMOS microelectrode array for the monitoring of electrogenic cells,” by F. Heer et al., Biosensors and Bioelectronics 20, 358-366 (March 2004) (“Heer”).

13. Appendix E to this declaration contains a copy of the Heer paper acquired from the Linda Hall Library. I compared Exhibit 1021 with Appendix E and find Exhibit 1021 to be a true and accurate copy of the Heer paper. On pages 10 and 11 of Appendix E, there is a date stamp with the date the issue was received. The

stamp states October 5, 2004. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 2004. Page 12 of Appendix E contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as December 12, 1990. Upon entry of that record into the catalog and subsequent processing of the issue containing the Heer article therein, this article would be locatable by keyword search. The 1990 MARC record indicates that the Linda Hall Library has been collecting issues of Biosensors and Bioelectronics since 1990. Thus, the library has been adding subsequent issues of Biosensors and Bioelectronics to its collection since 1990, including the Heer paper included in Volume 20 published in 2004. All of this indicates that the Heer paper was indexed and publicly available as of October 2004.

14. Exhibit 1022 is “A novel microfluidic impedance assay for monitoring endothelin-induced cardiomyocyte hypertrophy,” by Mo Yang et al., Biosensors and Bioelectronics (2006) (“Yang”).

15. Appendix F to this declaration contains a copy of the Yang paper acquired from the Linda Hall Library. I compared Exhibit 1022 with Appendix F and find Exhibit 1022 to be a true and accurate copy of the Yang paper. On page 7 of Appendix F, there is a date stamp with the date the issue was received. The stamp

states March 2007 (the exact date in March 2007 is not legible). Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in March 2007. Page 8 of Appendix F contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as December 12, 1990. Upon entry of that record into the catalog and subsequent processing of the issue containing the Yang article therein, this article would be locatable by keyword search. The 1990 MARC record indicates that the Linda Hall Library has been collecting issues of Biosensors and Bioelectronics since 1990. Thus, the library has been adding subsequent issues of Biosensors and Bioelectronics to its collection since 1990, including the Yang paper included in Volume 22 published in 2007. All of this indicates that the Yang paper was indexed and publicly available as of March-April 2007.

16. Exhibit 1023 is “Real Time Monitoring Primary Cardiomyocyte Adhesion Based on Electrochemical Impedance Spectroscopy and Electrical Cell-Substrate Impedance Sensing,” by Yiling Qiu et al., and published by February 2008, Analytical Chemistry, Vol. 80, No. 4, 990-996 (February 2008) (“Qiu”).

17. Appendix G to this declaration contains a copy of the Qiu paper acquired from the Linda Hall Library. I compared Exhibit 1023 with Appendix G and find

Exhibit 1023 to be a true and accurate copy of the Qiu paper. On pages 8-10 of Appendix G, there is a date stamp with the date the issue was received. The stamp states February 20, 2008. Based on my knowledge of library procedure and experience with serials, I believe this to mean that the issue was on the shelves and available to the public within two weeks of that date stamp in 2008. Page 11 of Appendix G contains the MARC record for this serial in the electronic catalog of the Linda Hall Library. The 008 field of the record has a date of creation for the serial record as July 27, 1947. Upon entry of that record into the catalog and subsequent processing of the issue containing the Qiu article therein, this article would be locatable by keyword search. The 1947 MARC record indicates that the Linda Hall Library has been collecting issues of Analytical Chemistry since 1947. Thus, the library has been adding subsequent issues of Analytical Chemistry to its collection since 1947, including the Qiu paper included in Volume 80 published in 2008. All of this indicates that the Qiu paper was indexed and publicly available as of February-March 2008.

18. I declare under penalty of perjury that the foregoing is true and correct.

Executed on August 30, 2024, at Jones Day, 2727 North Harwood Street, Suite 500, Dallas, TX 75201.



Cody A. Daniels

APPENDIX A

Accelerated Articles

Real-Time Monitoring of Morphological Changes in Living Cells by Electronic Cell Sensor Arrays: An Approach To Study G Protein-Coupled Receptors

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G protein-coupled receptors (GPCRs) constitute important targets for drug discovery against a wide range of ailments including cancer, inflammatory, and cardiovascular diseases. Efforts are underway to screen selective modulators of GPCRs and also to deorphanize GPCRs with unidentified natural ligands. Most GPCR-based cellular screens depend on labeling or recombinant expression of receptor or reporter proteins, which may not capture the true physiology or pharmacology of the GPCRs. In this paper, we describe a noninvasive and label-free assay for GPCRs that can be used with both engineered and nonengineered cell lines. The assay is based on using cell–electrode impedance to measure minute changes in cellular morphology as a result of ligand-dependent GPCR activation. We have used this technology to assay the functional activation of GPCRs coupled to different signaling pathways and have compared it to standard assays. We have used pharmacological modulators of GPCR signaling pathways to demonstrate the specificity of impedance-based measurements. Our data indicate that cell–electrode impedance measurements offer a convenient, sensitive, and quantitative method for assessing GPCR function. Moreover, the noninvasive nature of the readout offers the added advantage of performing multiple treatments in the same well to study events such as desensitization and receptor cross-talk.

G-protein-coupled receptors (GPCRs) constitute the largest family of plasma membrane proteins that are involved in signal transduction across the plasma membrane.^{1–3} These receptors play a prominent role in regulating a variety of physiological functions such as sight, taste, and smell, as well as fluid electrolyte balancing, cardiovascular function, and neurotransmission.² The vast majority of GPCRs couple to and activate heterotrimeric G proteins and subsequently stimulate second messenger systems. The heterotrimeric G proteins consist of a $G\alpha$ subunit and $G\beta/\gamma$ complex, both of which can activate downstream effectors depending on the receptors being activated.² More recently, it has also become apparent that GPCR signaling either through or in addition to heterotrimeric G proteins also activate the Rho family of small GTPases.^{3,4}

The Rho family of small GTPases, which include Rho, Rac, and CDC42, are well-characterized effectors of oncogenes, growth factor and adhesion-mediated signaling pathways^{5,6} and are not classically thought of as being key effectors for GPCRs. Rho family GTPases participate in a number of cellular processes, the main one being regulation and maintenance of specific structures within the actin cytoskeleton framework.⁶ GPCRs have been shown to modulate the actin cytoskeleton and hence cell morphology in a very specific manner depending on the Rho family GTPase being activated.^{6,7} The current view of the actin cytoskeleton is that of

- (1) Lefkowitz, R. J. *Trends Pharmacol. Sci.* **2004**, *25*, 413–422.
- (2) Pierce, K. L.; Premont, R. T.; Lefkowitz, R. J. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 639–650.
- (3) Hall, R. A.; Premont, R. T.; Lefkowitz, R. J. *J. Cell. Biol.* **1999**, *145*, 927–932.
- (4) Bhattacharya, M.; Babwah, A. V.; Ferguson, S. S. *Biochem. Soc. Trans.* **2004**, *32*, 1040–1044.
- (5) Bishop, A. L.; Hall, A. *Biochem. J.* **2000**, *348*, 241–255.
- (6) Etienne-Manneville, S.; Hall, A. *Nature* **2002**, *420*, 629–635.
- (7) Jaffe, A. B.; Hall, A. *Annu. Rev. Cell. Dev. Biol.* In press.

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a dynamic and plastic system that is a reflection and manifestation of the intracellular signaling and not simply a static structure designed to maintain cellular architecture.⁸ Since GPCRs couple to the actin cytoskeletal network and induce very defined morphological changes, it is possible to harness this information as a functional and biologically relevant readout for GPCRs.

Therefore, we have designed electronic cell sensor arrays embedded in the bottom of the well of microtiter plates that are capable of measuring minute changes in cell morphology.^{9–11} The electronic sensors measure changes in cell–substrate impedance as a result of the disruption of the ionic environment due to the presence of cell and cell morphology dynamics. The main advantages offered by using cell–substrate impedance and cell morphology as a readout are that both exogenously expressed and endogenous receptors can be assayed without the need for engineering the cell with promiscuous G proteins and reporters or labeling the cells with dyes. In addition, since the readout is noninvasive, multiple stimulations with the same ligand or different ligands can be performed to assess events such as desensitization and receptor cross-talk. Finally, another major aspect of using cell–substrate impedance and cell morphology as a readout is that potentially all GPCRs, regardless of the signaling pathways, can be functionally monitored.

In this paper, we explore the utility of using cell–substrate impedance as a quantitative and functional readout for GPCRs. We tested both exogenously expressed and endogenous receptors coupled to different signaling pathways and performed side-by-side comparison with standard assays such as IP₃ and cAMP measurements. The cells were continuously monitored using a real-time cell electronic sensing (RT-CES) system that we have developed. Using molecular, cellular, and biochemical assays, we demonstrate that the impedance readout correlates with specific changes in cell morphology and activation of signaling pathways and interfering with these pathways using pharmacological inhibitors abolishes the impedance readout. Furthermore, we have used cell–substrate impedance-based measurement of cell morphology to assay and characterize inverse agonists for the histamine H1 receptor and to assess its desensitization.

MATERIALS AND METHODS

Cell Culture. All the cells used in this study were purchased from ATCC unless indicated otherwise. Cells are cultured in a standard humidified incubator at 37 °C with 5% CO₂/95% air. HeLa and N1E-115 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 IU/mL penicillin and 100 µg/mL streptomycin. CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 IU/mL penicillin and 100 µg/mL streptomycin. Cell lines stably expressing human histamine receptor 1 (H1), human dopamine receptor 1 (D1), and human vasopressin 1a receptor (V1a) in CHO-K1 cells were obtained from Euroscreen and maintained in Ham's/F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100

IU/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418. Cell lines stably expressing human 5-hydroxytryptamine receptor 1A (5-HT1A) and 5-hydroxytryptamine receptor 2B (5-HT2B) in CHO-K1 cells were obtained from Euroscreen and maintained in Ultra-CHO medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418.

Reagents. All the reagents were purchased from Sigma unless indicated otherwise. Loratidine, mirtazepine, mepyramine, triprolidine, tiotidine, clemastine, dimethidene, doxepin, ketotifen, SFK 38393, and 8-OH-DPAT were purchased from Tocris.

RT-CES Experimental Setup. The detailed experimental procedures have been described previously.¹¹ Briefly, 95 µL of media was added to wells in 16× E-plates to obtain background readings followed by the addition of 100 µL of cell suspension containing the indicated number of cells. The E-plates containing the cells were allowed to incubate at room temperature for 10 min before being placed on the device station in the incubator for continuous recording of impedance as reflected by cell index (CI, see detailed definition below). The cells were allowed to attach and spread typically for 6–8 h to reach a stable baseline before the addition of agonists. Typically, 5 µL of 40× stock solution of agonist was gently added to the well and recording was resumed. For pharmacological and mechanistic studies, antagonists or inhibitors were added to the cells 5–10 min prior to agonist application. The results were expressed by normalized CI, unless indicated otherwise, which are derived from the ratio of CIs before and after the addition of the compounds. For concentration-dependent study, maximal response of a given concentration of the compound was used to plot the concentration-dependent curve and EC₅₀ or IC₅₀ were calculated by Prism.

Impedance and Cell Index Measurements by RT-CES System. The electrodes in the E-plates are made out of thin gold film using lithographical methods. Gold is deposited on glass slides and is then patterned to form electrode arrays. The “circle on line” design used in this technology was chosen because it allows for maximal sensitivity for the detection of the cells with relatively uniform distribution of the electric field while maximizing the coverage area of each well; the electrodes cover ~75% of the total area of the well. Impedance measured between electrodes in an individual well depends on electrode geometry, ionic concentration in the well, and whether there are cells attached to the electrodes. In the absence of the cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of the cells, cells attached to the electrode sensor surfaces will alter the local ionic environment at the electrode/solution interface, leading to an increase in the impedance. The more cells there are on the electrodes, the larger the increase in cell–electrode impedance. Furthermore, the impedance change also depends on cell morphology and the extent to which cells attach to the electrodes.

To quantify cell status based on the measured cell–electrode impedance, a parameter termed cell index is derived, according to

$$CI = \max_{i=1, \dots, N} \left(\frac{R_{\text{cell}}(f_i)}{R_b(f_i)} - 1 \right)$$

(8) Bhawe, G.; Gereau, R. W. *J. Neurosci.* **2003**, *23*, 577–579.

(9) Xing, J. Z.; Zhu, L.; Jackson, J. A.; Gabos, S.; Sun, X. J.; Wang, X. B.; Xu, X. *Chem. Res. Toxicol.* **2005**, *18*, 154–161.

(10) Abassi, Y. A.; Jackson, J. A.; Zhu, J.; O'Connell, J.; Wang, X.; Xu, X. *J. Immunol. Methods* **2004**, *292*, 195–205.

(11) Solly, K.; Wang, X.; Xu, X.; Strulovici, B.; Zheng, W. *Assay Drug Dev. Technol.* **2004**, *2*, 363–372.

where $R_b(f)$ and $R_{cell}(f)$ are the frequency-dependent electrode resistances (a component of impedance) without cells or with cell present, respectively. N is the number of the frequency points at which the impedance is measured. Thus, CI is a quantitative measure of the status of the cells in an electrode-containing well. Under the same physiological conditions, more cells attached onto the electrodes leads to a larger $R_{cell}(f)$ value, leading to a larger value for cell index. Furthermore, for the same number of cells present in the well, a change in the cell status such as morphology will lead to a change in the cell index. For example, an increase in cell adhesion or cell spreading leads to larger cell-electrode contact area, which will lead to an increase in $R_{cell}(f)$ and thus a larger value for cell index. A "normalized cell index" at a given time point is calculated by dividing the cell index at the time point by the cell index at a reference time point. Thus, the normalized cell index is 1 at the reference time point.

Fluorescence Microscopy. H1/CHO and V1a/1321-N1 cells were seeded in 16-well Lab-tek chamber slides and allowed to attach and spread for 24 h at 37 °C. The cells were stimulated with 100 nM histamine or μ M vasopressin for 5 min, followed by fixation in 4% paraformaldehyde for 10 min and permeabilization in PBS containing 0.2% Triton X 100 for 10 min. After washing, the fixed cells were blocked in PBS containing 0.5% BSA for 30 min. The cells were stained with phalloidin conjugated with FTIC (10 μ g/mL final concentration in blocking buffer) and a monoclonal anti-paxillin ascites fluid (1:500 final dilution in blocking buffer) for 1 h. The cells were washed 3 times with PBS, and the cells stained with anti-paxillin ascites fluid were further stained with goat-anti-mouse IgG conjugated to TRITC (Sigma, 20 μ g/mL final dilution) for 1 h. All incubations were carried out at room temperature. The slides were mounted, visualized, and imaged using a Nikon E400 epifluorescence microscope and Nikon ACT software.

Immunoprecipitation and Western Blotting Analysis for Phosphorylated Paxillin. H1/CHO cells were stimulated with vehicle or 20 nM histamine for 5 min. The cells were lysed with RIPA buffer containing phosphatase and protease inhibitors, and the lysate was centrifuged for 10 min at 14 000 rpm in a Beckman microcentrifuge. The soluble lysate was incubated with 2.5 μ g of anti-paxillin monoclonal antibody and immunoprecipitated with the addition of protein A-Sepharose. The immunoprecipitates were washed, eluted in 2 $\times$ SDS sample buffer, and fractionated on 4–20% SDS-PAGE gel (Invitrogen). The gel was transferred to a nitrocellulose membrane and immunoblotted with either PY-20 HRP (Santa Cruz Biotech) or anti-paxillin antibody. The blot was developed using SuperSignal West Dura Extended Duration Substrate (Pierce), and images were captured by a CCD camera (UVP BioImaging System).

IP₃ and cAMP Assays. To quantify the functional activities of H1/CHO cells, a modification of the described method¹² based on the measure of the depletion of [³H]-phosphatidylinositol was used. Briefly, 2.8×10^6 cells were loaded overnight with [2-³H-(N)]myo-inositol (10 nCi/plate), washed 3 times, and detached with PBS-EDTA. A total of 2.5×10^5 cells were resuspended in 400 μ L of LiCl buffer (121.5 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 24.93 mM Tris-HCl, 15 mM glucose, and

10 mM LiCl, pH 7.4) and incubated with 100 μ L of histamine at different concentrations for 30 min. The reaction was stopped by adding 500 μ L of methanol/HCl (88 mL of ethanol/12 mL 1 M HCl) and quick frozen at -20 °C. The cells were filtered on GF/B filters preincubated in 0.1% PEI, and [2-³H(N)]myo-inositol was counted in a TriCarb counter. Results shown are expressed as a decrease of membrane-bound [³H]PI content (in counts/min).

The effects of increasing concentration of SKF 38393 on D1/CHO-K1 cells were tested by seeding cells the day before the experiment (2.5×10^4 /well in a 96-well plate). The cells were incubated in KRH + IBMX buffer for 15 min at 37 °C. After this preincubation, increasing amounts of SKF 38393 were added to the wells and incubated for 20 min at 37 °C. The reaction was stopped by removal of the supernatant, and 100 μ L of lysis buffer was added on each well. The plate was incubated for 30 min at 37 °C and finally spun down for 3 min at 1200 rpm. The supernatant were recovered, and cAMP concentrations were determined using a Tropic kit (Applied Biosystems), according to manufacturer's specifications.

To assess the functional activation of 5-HT_{1A} receptor, 5-HT_{1A}/CHO cells were grown for 20 h in media without antibiotics and detached by PBS-EDTA. After centrifugation, cells were suspended at 6.25×10^5 cells/mL in KRH-IBMX (5 mM KCl, 1.25 mM MgCl₂, 124 mM NaCl, 25 mM HEPES pH 7.4, 13.3 mM glucose, 1.25 mM KH₂PO₄, 1.45 mM CaCl₂, and 0.5 g/L BSA supplemented with 1 mM IBMX). The 96-well plates (Costar) are then successively filled with KRH-IBMX, cells (7.5×10^3 cells/well), increasing concentrations of agonist (diluted in KRH-IBMX), and forskolin (10 μ M). The plate was then incubated for 30 min at room temperature. After addition of the lysis buffer, cAMP concentrations are estimated by an HTRF kit from Cis-Bio International.

RESULTS

Principle of Cell Electrode Impedance and Measurement of Cell Morphological Dynamics in Response to GPCR Activation. We have designed interdigitated microelectrodes that are embedded in the bottom of the wells of microtiter plates (E-plates) that are used in conjunction with an RT-CES system.^{10,11} As illustrated in Figure 1, in the absence of cells, the impedance value (Z) is derived from the electrode/solution interface (Z_0). The presence of cells on the electrode will affect the local ionic environment as well as the electrode/solution interface leading to an increase in electrode impedance. The impedance difference between the presence and absence of cells in the well reflects the pure impedance due to the presence of the cells (Z_{cell}). The addition of the agonist for different GPCRs will affect cell morphology in a very specific manner depending on the pathways being activated, and further change in impedance is a reflection of the morphological dynamics that are induced by the ligand ($Z_{activated\ cell}$). Because the readout can be sampled in as little as 50 ms/well, the rate of change, magnitude, and duration of the response can be precisely measured.

To demonstrate the utility of impedance measurement of cell morphology as a functional readout for GPCR activation, CHO-K1 cells expressing the human H1 histamine receptor (H1/CHO) and 1321-N1 cells expressing the human vasopressin receptor (V1a/1321-N1) were seeded on the E-plates and stimulated with histamine and vasopressin, respectively (Figure 2). Both histamine

(12) Cussac, D.; Newman-Tancredi, A.; Sezgin, L.; Millan, M. J. *Naunyn-Schmiedeberg Arch. Pharmacol.* **2000**, *361*, 569–572.

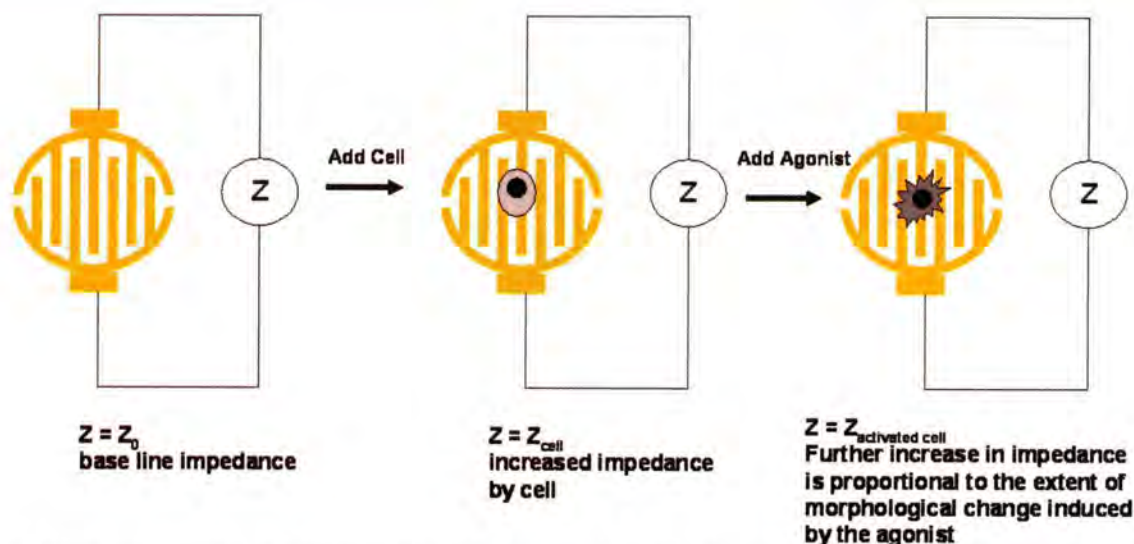


Figure 1. Schematic representation of microelectrodes and the principle of utilizing cell–electrode impedance to noninvasively measure GPCR-mediated modulation of cell morphology. In the absence of cells, the baseline impedance (Z_0) value is determined by the software. Addition of cells to the sensor microelectrodes leads to changes in impedance signal (Z_{cell}) that is directly proportional to the number of cells seeded on the sensors. Agonist-mediated stimulation of GPCRs expressed on the cell surface leads to transient changes in cell morphology that further increase or decrease the impedance value ($Z_{\text{activated cell}}$) depending on the activated signaling pathways.

and vasopressin induced an immediate (within 5 min) and transient increase in impedance, which is represented as normalized CI. The maximal response for H1/CHO cells was at 40 min after histamine addition (Figure 2A), while it was at 36 min after vasopressin addition for V1a/1321-N1 cells (Figure 2C). Both H1 receptor and V1a receptor act through Gq and can modulate the actin cytoskeleton and its regulatory proteins such as focal adhesion kinase and paxillin.^{13–15} To determine whether histamine- and vasopressin-induced increases in normalized CI correlate with modulation of the actin cytoskeleton and its associated proteins, H1/CHO cells and V1a/1321-N1 cells were stimulated with histamine and vasopressin, respectively, fixed, and stained with FITC–phalloidin and anti-paxillin mAb. Histamine treatment of H1/CHO cells led to an immediate (5 min) induction of membrane ruffles and translocation of paxillin to the site of membrane ruffles, which is indicative of active actin remodeling (Figure 2B, lower right panel). Similarly, vasopressin also induced formation of membrane ruffles and translocation of paxillin to these sites (Figure 2D, lower right panel). Furthermore, as shown previously, histamine-mediated stimulation of H1 receptor leads to tyrosine phosphorylation of paxillin, a major regulator of actin cytoskeleton (Figure 2E). In summary, impedance-based measurements of GPCR-mediated actin cytoskeleton dynamics and morphological changes can serve as a useful readout for ligand-mediated GPCR activation.

Specificity of Ligand-Mediated Changes of Impedance as a Functional Readout for GPCR Activation. To establish the specificity of impedance measurements represented as normalized CI as a functional readout for GPCR activation, we undertook a multifaceted approach including utilization of specific antagonist,

pathway blockers, and pathway modulators. Starting at the level of the H1 histamine receptor in H1/CHO cells, a number of selective H1 receptor antagonists were screened and ranked according to their relative potencies. As shown in Figure 3A, all H1 receptor antagonists blocked histamine-induced CI increases in H1/CHO cells in a concentration-dependent manner. Plotting the peak responses against concentrations of antagonists allows for the calculation of IC_{50} s (see caption of Figure 3. for IC_{50} s). No significant effect of tiotidine, a selective H2 receptor antagonist, was found at the highest concentration used (100 μM).

It has been well established that activation of Gq leads to stimulation of phospholipase C and consequently protein kinase C (PKC) pathways. Stimulation of Gs activates adenylate cyclase and hence increases cAMP while Gi is negatively coupled to cAMP.² Therefore, we attempted to test the specificity at the pathway level. First, we demonstrated that blocking the PKC pathway by bisindolylmaleimide, a specific inhibitor of PKC, completely abolished histamine-induced CI increases (Figure 3B) in H1/CHO cells, indicating the involvement of the PKC pathway. Second, dopamine receptor 1, overexpressed in CHO-K1 cells (D1-CHO, coupled to Gs) was stimulated with its natural ligand dopamine in addition to forskolin. As shown in Figure 3C, dopamine and forskolin, an adenylate cyclase activator that bypasses the receptor and artificially activates adenylate cyclase, lead to CI increases with similar kinetics and comparable amplitudes, indicating that cAMP production may result in increases in CI. Finally, to demonstrate the specificity of serotonin (5-hydroxytryptamine, 5-HT) on serotonin receptor (5-HT1A receptor) overexpressed in CHO-K1 cell (5-HT1A/CHO, coupled to Gi), the cells were pretreated with pertussis toxin, a potent and selective inhibitor of Gi¹⁶ before 5-HT was added. As shown in Figure 3D (left panel), pertussis toxin pretreatment selectively blocks serotonin-induced 5-HT1A activation but does not affect serotonin-induced stimulation of 5-HT2B cells, which is coupled

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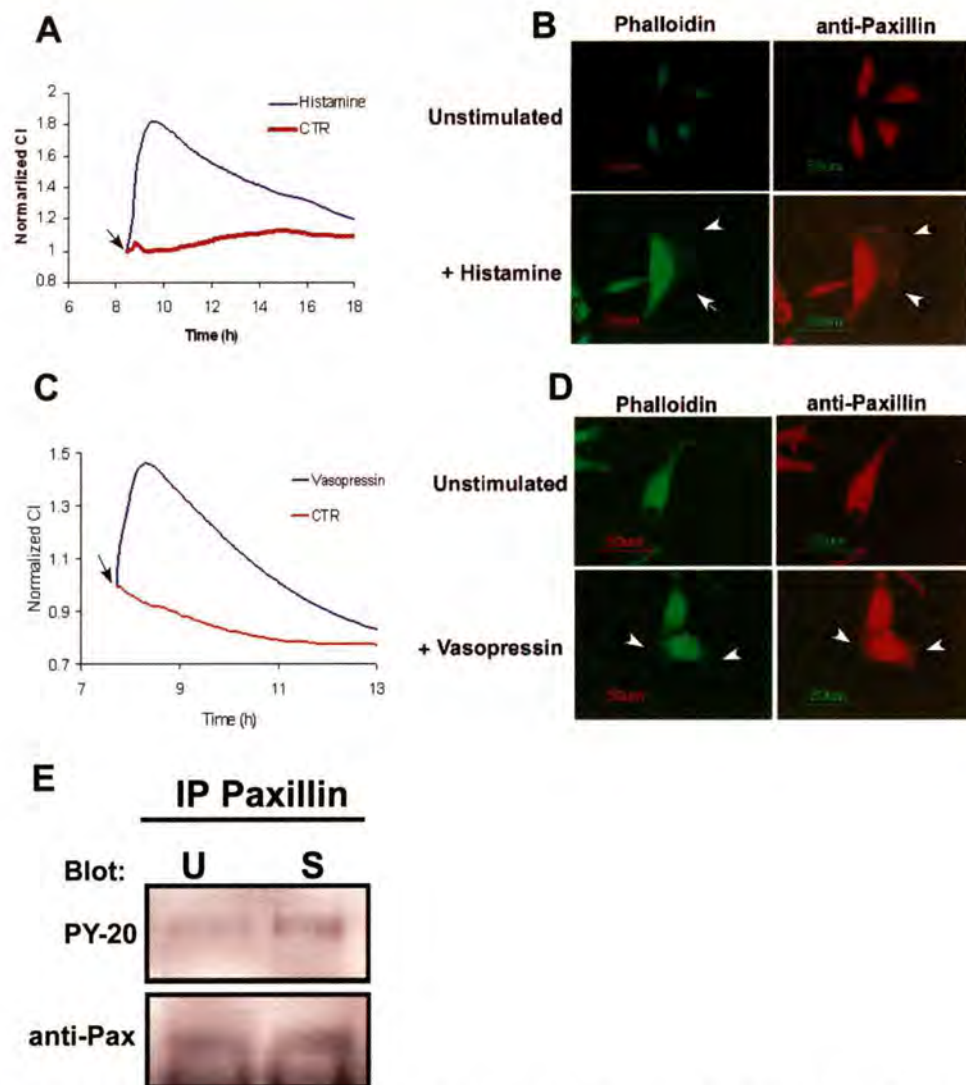


Figure 2. Correlation of agonist-induced increases of CI and cell morphology dynamics. (A) H1/CHO cells were seeded on cell sensor microelectrodes and stimulated with various concentrations of histamine (indicated by the arrow). Cell sensor impedance as displayed by normalized CI was continuously monitored every 3 min for the duration of the experiment. (B) H1/CHO cells were seeded on 16-well Lab-tek chamber slides and stimulated with vehicle or 100 nM histamine for 5 min. After stimulation, the cells were fixed and processed as described in Materials and Methods. The arrows indicate the site of membrane ruffles and paxillin translocation as a result of histamine treatment. The scale bar is in micrometers. (C) V1a/1321-N1 cells were seeded on cell sensor microelectrodes and stimulated with vehicle or 1 μ M vasopressin. Cellular response was continuously monitored every 3 min as described above. (D) V1a/1321-N1 cells were seeded on 16-well Lab-tek chamber slides and stimulated with vehicle or 1 μ M vasopressin for 5 min. After stimulation, the cells were fixed and processed as described in section of methods. The arrows indicate the site of membrane ruffles and paxillin translocation as a result of vasopressin treatment. The scale is in micrometers. (E) H1/CHO cells were stimulated with 100 nM histamine for 15 min. After the stimulation, the cells were processed as described in Materials and Methods. Upper panel shows the phosphorylated paxillin detected by PY-20 antibody in unstimulated (U) and stimulated (S) cells. Lower panel shows the total paxillin after immunoprecipitation.

to Gq and is pertussis toxin insensitive (Figure 3D, right panel). Taken together, these results strongly support the notion that cell-electrode impedance measurement as displayed by CI is dependent on the stimulation of specific signal transduction pathways mediated by a specific receptor/ligand pair of GPCRs.

Monitoring Activation Kinetics of Exogenous and Endogenous GPCRs in Live Cells: Comparison with Traditional Assays for EC_{50} Values. To measure functional activation of GPCRs by cell-electrode impedance measurement, both recombinant GPCRs and endogenous GPCRs were tested with their respective agonists. For recombinant GPCRs, three representative categories of GPCRs were tested by the RT-CES system, namely, H1/CHO (Gq), D1/CHO (Gs) and 5-HT1A/CHO (Gi). The cells

were seeded on E-plates and treated with the indicated concentrations of either respective natural agonist (histamine, Figure 4A, upper panel) or receptor subfamily selective agonists (SKF 38393, D1 selective, Figure 4A, middle panel and 8-OH-DPAT, 5-HT1A selective, Figure 4A, lower panel). For each treatment, the cellular responses from four agonist concentrations are shown. Ligand application led to an immediate and sharp increase (H1/CHO and D1/CHO) in normalized CI, which reached maximal response between 30 min and 1 h depending on the receptors. Plotting the maximal response against each concentration allows for the calculation of EC_{50} for each agonist as shown in Figure 4B. To further validate these findings, we performed functional GPCR assays using standard methods including a [^{3} H]-phosphatidyl-

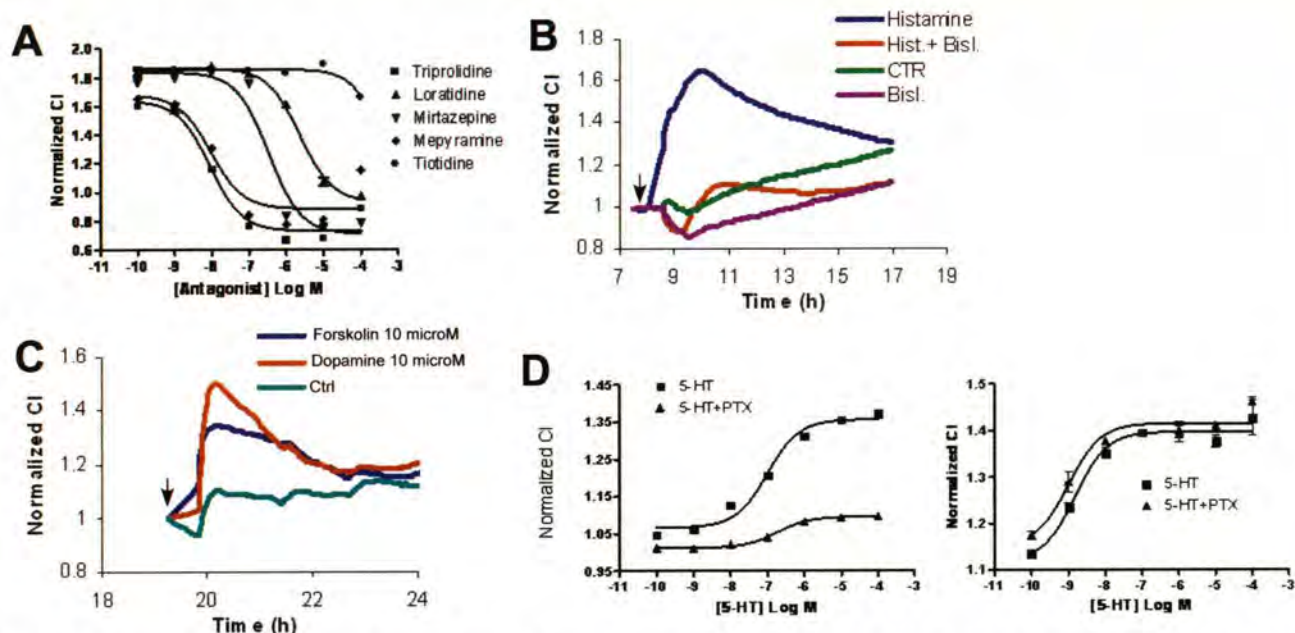


Figure 3. Specificity characterization of GPCR-mediated CI increases in three CHO cell lines stably expressing three representative GPCRs. (A) Selective H₁ receptor antagonists but not H₂ receptor antagonist (tiotidine) blocked histamine-induced CI increases in a concentration-dependent manner. IC_{50} s (in nM) for triprolidine, loratidine, mirtazapine, and mepyramine were 9, 24 000, 343, and 9, respectively. IC_{50} s (in nM) for brompheniramine, clemastine, dimethidene, doxepin, and ketotifen were 16, 5, 394, 8, and 2, respectively (curves not shown). IC_{50} for tiotidine was greater than 100 μ M (microM). (B) Complete block of histamine-induced CI increase in H₁/CHO by PKC inhibitor bisindolylmaleimide. (C) Comparison of magnitude and duration of CI increases induced by dopamine and adenylate cyclase activator forskolin in D₁/CHO cells (coupled to G_s). (D) Pertussis toxin pretreatment abolished 5-HT-induced CI increases only in 5-HT_{1A}/CHO cells (coupled to G_i; left panel) but not in 5-HT_{2B}/CHO cells (coupled to G_q; right panel).

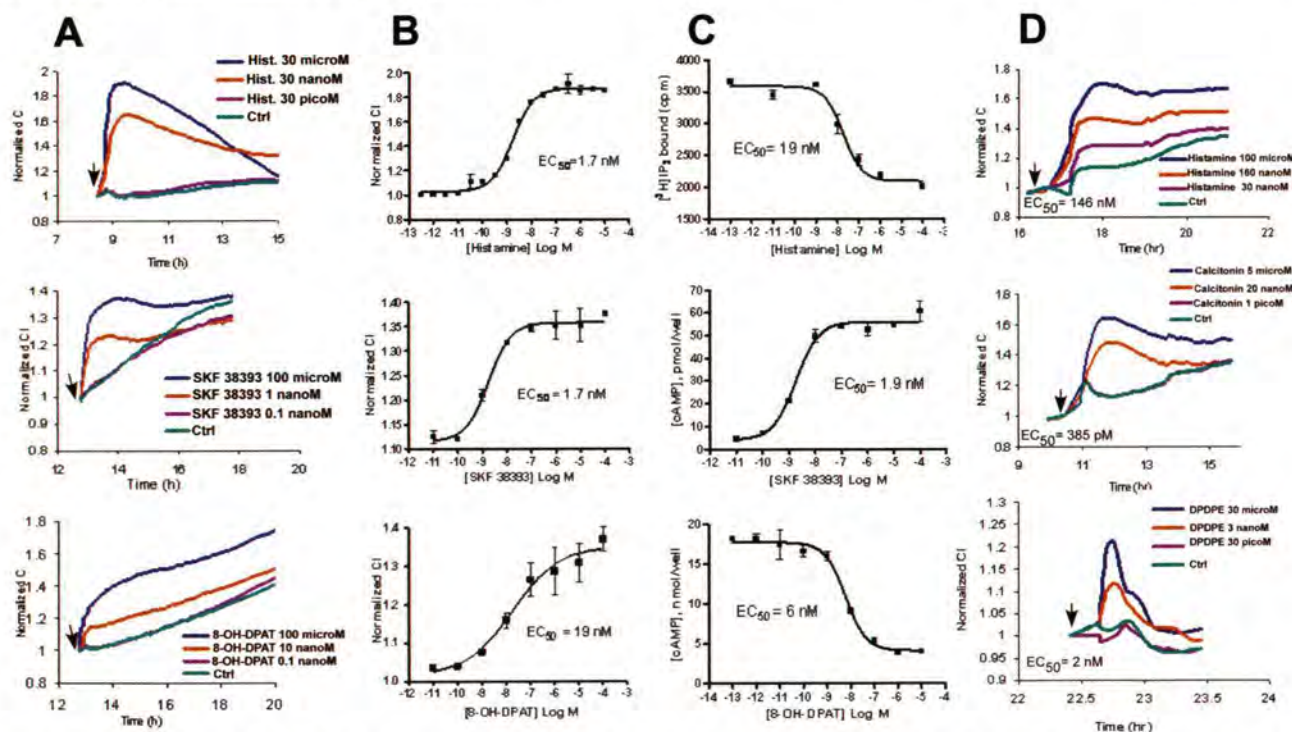


Figure 4. Comparison of RT-CES system with standard GPCR functional assays to obtain EC_{50} s of agonists in exogenous GPCRs and application for endogenous GPCRs. (A) Representative traces of CI of H₁/CHO (upper panel), D₁/CHO (middle panel), and 5-HT_{1A}/CHO (lower panel) cells were stimulated by their ligands histamine, SKF 38393 and 8-OH-DPAT (arrows), respectively. The cells were continuously monitored. (B) Plotting the peaks of normalized CI responses versus corresponding agonist concentrations allows for the generation of concentration-dependent curves and calculation of EC_{50} s as shown in each panel. (C) Concentration-dependent curves and EC_{50} s generated from standard GPCR assays. Histamine on H₁/CHO cells (upper panel), SKF 38393 on D₁/CHO cells (middle panel), and 8-OH-DPAT on 5-HT_{1A}/CHO cells (lower panel). (D) Measurement of activation of three representative endogenous GPCRs by RT-CES system. Response profiles and EC_{50} s for each agonist/cell pair are shown for histamine/HeLa cells (upper panel), calcitonin/CHO cells (middle panel), and DPDPE/N1E-115 cells (lower panel), which express endogenous H₁ histamine receptor, calcitonin receptors, and opioid δ receptors, respectively.

nositol depletion assay for H₁/CHO (Figure 4C, upper panel), cAMP measurement for D₁/CHO (Figure 4C, middle panel) and

inhibition of forskolin-induced cAMP for 5-HT_{1A}/CHO (Figure 4C, lower panel). EC_{50} values obtained by cell-electrode imped-

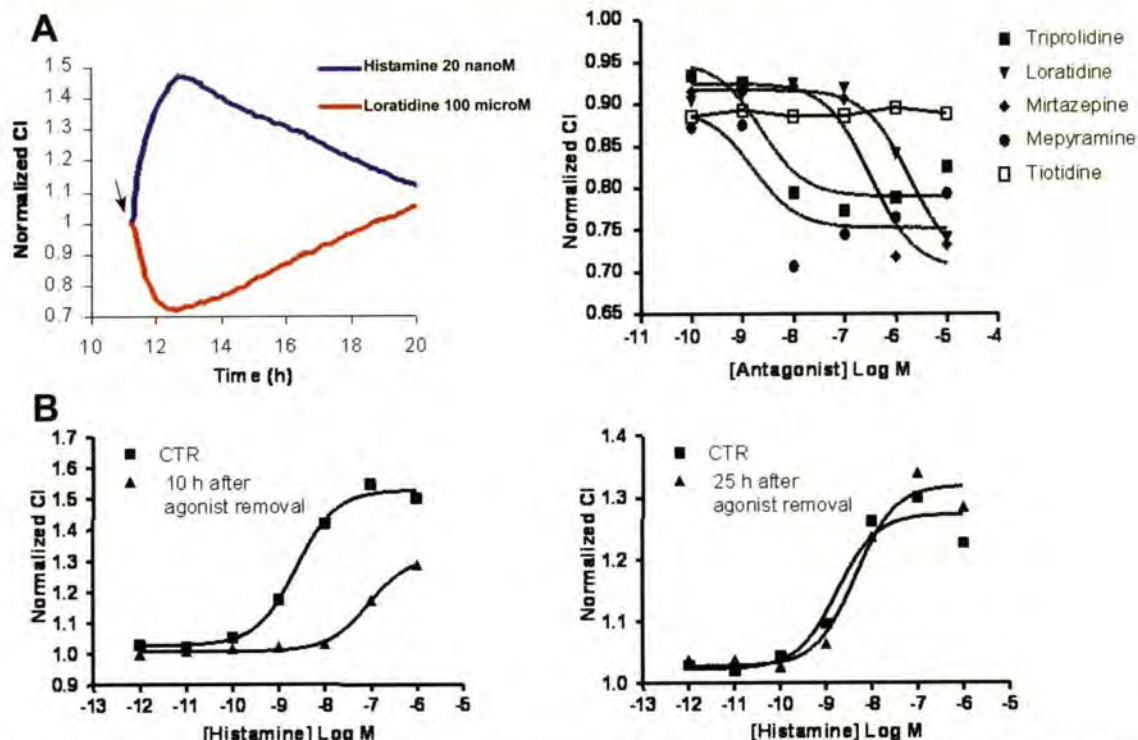


Figure 5. Utilization of RT-CES system to characterize inverse agonists and study desensitization of GPCRs. (A) Selective H1 antagonist loratidine (left panel) or other H1 receptor antagonists (right panel) alone were incubated with H1/CHO cells. Plotting the lowest values of normalized CI responses versus corresponding agonist concentrations allows for the generation of concentration-dependent curves. Tiotidine, a selective H2 antagonist did not affect CI. (B) Dynamics of H1 receptor desensitization. Ten hours after the initial activation by histamine, the medium of H1 cells was removed and the cells were washed twice and replaced with fresh medium. Ten (left panel) and 25 h (right panel) after removal of media, various concentrations of histamine were reintroduced to the cells and responses of impedance were monitored continuously. Concentration-dependent curves were generated, and EC_{50} s were calculated by plotting the maximal responses versus the concentrations of histamine.

ance measurements (Figure 4B) are comparable to the EC_{50} values obtained by the traditional assays (Figure 4C) indicating that the assays are equally sensitive.

One of the challenges encountered by current label-based assays such as calcium measurements is that the cells have to be engineered to express promiscuous G proteins coupled to the calcium pathway.¹⁷ Alternatively, receptors may need to be overexpressed in order to obtain sufficient measurable signal.¹⁸ All these artificial manipulations may ease the burden of screening large sets of compounds; however, physiological and pharmacological relevance of the findings will need to be supplemented with additional experiments. The number of assays available to measure the functional activation of endogenous GPCRs in their natural and physiologically relevant settings is limited. To determine the utility of impedance-based measurements in monitoring activation of endogenous receptors, three different cell lines expressing endogenous levels of histamine receptor, calcitonin receptor, and δ opioid receptor were selected. We tested histamine on HeLa cells (Gq), calcitonin on CHO cells (Gs), and DPDPE (selective δ opioid receptor agonist, Gi) on N1E-115 mouse neuroblastoma cells. Similarly, all the ligands generated transient increases of CI (Figure 4D). Peak CIs of each concentration of the agonist were chosen to plot the concentration-dependent curves and to extrapolate the EC_{50} s for histamine (Figure 4D,

upper panel) calcitonin (Figure 4D, middle panel), and DPDPE (Figure 4D, lower panel). In summary, these results indicate that cell-electrode impedance measurements can be used to functionally assess both endogenous and exogenous GPCRs dynamically without the need for labeling or engineering the cells to express reporter proteins. Furthermore, receptors coupled to the three common pathways, Gq, Gs, and Gi, can be assayed by the same technology platform.

Application of the RT-CES System To Characterize Inverse Agonists and To Study Desensitization of H1/CHO. Interestingly, the majority of the H1 receptor antagonists have been shown to stabilize the inactive state and serve as inverse agonists of the H1 histamine receptor^{19–21} by decreasing the basal levels of IP_3 and calcium.²² Incubation of H1/CHO with H1 receptor “antagonist”, loratidine alone (Figure 5A, left panel) or other H1 receptor antagonists (Figure 5A, right panel) lead to a dose-dependent decrease in CI. Incubation of the same compounds with CHO-K1 cells had no effect (data not shown). These results strongly support the notion that cell morphology is a very sensitive indicator, and manifestation of intracellular signaling and impedance measurements of cell morphology can be potentially used to discover and characterize inverse agonists of different GPCRs.

Last, we took advantage of the noninvasive nature of impedance readout to assess the desensitization of H1 histamine receptor. Ten hours after the initial histamine stimulation, the media were removed and the cells were washed. Various concentrations of histamine were reintroduced to the cells 10 and 25 h after the

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removal of the media containing histamine, respectively. As shown in Figure 5B, 10 h (left panel), after the removal of histamine, the magnitudes to histamine restimulation were 57% of the control with a 37-fold (89 vs 2.4 nM) increase of EC₅₀ and 25 h (right panel) afterward with comparable magnitudes and 2.5-fold (4.5 vs 1.8 nM) increases of EC₅₀. These results clearly demonstrated that RT-CES system is a simple and effective means to study receptor desensitization.

In summary, we have shown that impedance measurements can be used for pharmacological characterization of GPCR. According to our data, impedance readout is as sensitive as some of the standard functional assays for GPCR activation, such as IP₃ and cAMP measurements. The major advantages of impedance as a functional readout for GPCR activation are that the cells do not need to be labeled or engineered with promiscuous G proteins and reporters. Therefore, cells expressing both recombinant and endogenous receptors can be assayed. Furthermore, potentially all classes of GPCRs, regardless of the pathways they are coupled to, can be assayed on the same technology platform. In addition, since the readout is noninvasive, multiple stimulations with ligands, agonists, or antagonists can be conducted in the same well over time to assess receptor cross-talk or desensitization.

DISCUSSION

In addition to the canonical signaling pathways mediated by various G proteins, most GPCRs also have the capacity to modulate the actin cytoskeleton through the Rho family GTPases.^{3,8} The manner in which the actin cytoskeleton and hence cell morphology is modulated by GPCRs largely depends on the Rho family member(s) being activated. Each member of the Rho family GTPase affects the actin cytoskeleton in a very specific manner.⁷ For example, activation of Rho induces stress fiber formation and cellular contraction and rounding; Rac is mainly responsible for membrane ruffles and lamellipodia formation, which are extensions of the plasma membrane; CDC42, another member of the Rho family GTPases, induces the formation of filipodia, which are also extensions of the plasma membrane but distinct from lamellipodia.^{5,6} In this paper, we have described a noninvasive and label-free method based on measurement of cell-electrode impedance. The RT-CES system has the capacity to quantitatively measure minute changes in cell morphology. Due to the kinetic aspects of the measurements, agonist stimulation of GPCRs leads to receptor-specific profiles that depend on the signaling pathways being activated.

To establish the specificity of using cell morphology changes in conjunction with impedance measurements as functional readout for GPCRs, we screened several well-established antagonists of the H1 histamine receptor. According to our data, the histamine H1 receptor antagonists were able to dose-dependently and selectively block histamine-mediated stimulation of H1/CHO cells, whereas selective H2 (tiotidine) receptor antagonist did not have any effect, demonstrating the specificity of the response (Figure 3A). In addition, we were able to demonstrate that

selective PKC inhibitor, bisindolylmaleimide, was able to completely block the H1/CHO cell response to histamine stimulation, indicating that the PKC pathway plays an important role in H1 receptor signaling pathways leading to morphological changes (Figure 3B). This finding is corroborated by earlier findings that PKC plays an important role in histamine H1 receptor-mediated responses such as cytokine secretion and activation of transcription.^{23,24} The specificity of the D1/CHO receptor activation, which is coupled to Gs activation of adenylate cyclase and production of cAMP, was demonstrated by using forskolin to artificially activate adenylate cyclase and cAMP production (Figure 3C). The response profiles of D1/CHO to forskolin were very similar to those by dopamine in terms of amplitude and duration (Figure 3C), indicating that the cAMP pathway may be responsible for D1 receptor-induced cellular response on the electronic sensors. Finally, the specificity 5-HT1A/CHO (coupled to Gi) upon stimulation with 5-HT was demonstrated by using pertussis toxin (PTX), a specific inhibitor of Gi. PTX completely blocked 5-HT stimulation of 5-HT1A/CHO cell impedance response (Figure 5C, left panel) while 5-HT-induced responses in 5-HT2B/CHO (coupled to Gq) were not affected by PTX treatment (Figure 5C, right panel).

We have taken several approaches to demonstrate the utility and versatility of impedance measurements as a functional readout for GPCRs. First, we have demonstrated that different recombinantly expressed GPCRs coupled to the Gq, Gs, and Gi pathways can be monitored in an agonist-dependent manner in live cells using the RT-CES system (Figure 4). For histamine-mediated stimulation of H1/CHO cells, we have shown that the impedance signal generated corresponds to concurrent changes in cell morphology in the form of membrane ruffles, being indicative of Rac activation. Furthermore, histamine stimulation of H1/CHO cells is also accompanied by concomitant phosphorylation of paxillin, an actin-binding protein that is known to be involved in modulation of the actin cytoskeleton.²⁵ The versatility of the impedance measurements as a readout for GPCRs is demonstrated by the fact that receptors linked to three different pathways can be functionally monitored on the same system. Typically, several different technologies including those capable of measuring calcium or IP₃ and cAMP would be required to functionally assess all these receptors. Furthermore, our data indicate that measurement of cell sensor impedance upon agonist addition is as sensitive as traditional assays such as IP₃ and cAMP measurements (Figure 4), which are reflected by similar EC₅₀ values.

The other major benefit of impedance measurements with respect to GPCRs is their capability to monitor activation of endogenous GPCRs in live cells without the need for labels or reporters (Figure 4D). This is a much needed development in the GPCR assay field because it allows for the monitoring of GPCRs in their natural and relevant cell lines or even primary cells. Physiological relevance of current assays that employ labels, reporters, and overexpression of GPCRs needs to be interpreted with much caution because it may not necessarily reflect the actual signaling scheme of the receptors in their natural states.¹⁸ Overexpression of receptors can disrupt the stoichiometry of the

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receptor with respect to downstream signaling and can lead to aberrant measured responses. Furthermore, some receptors may be difficult to overexpress in cells in order to assess their function. Moreover, another pitfall with overexpression is the realization that certain receptors in their natural and physiological setting form heterooligomers with other receptors with very distinct pharmacological and regulatory properties.^{26,27} Therefore, while overexpression in a heterologous cell line may be convenient for the generic identification of agonists and antagonists, the signaling pathways employed in a heterologous system such as CHO cells may vary significantly when compared to a primary cell such as a neuron.

Interestingly, while screening H1 receptor antagonist, we observed that the antagonists alone dose-dependently decreased the baseline impedance values in H1/CHO cells (Figure 5A) but not in parental CHO cells (data not shown). This observation indicated to us the possibility that H1 antagonists may act as inverse agonists of the H1 receptor rather than a pure antagonist. Indeed, it has been shown in the literature that most H1 antagonists do function as inverse agonists through the H1 receptor serving to downmodulate basal signaling such as IP₃ production.^{19,21} This is an important observation and further

supports the premise that cellular morphology is a very sensitive and precise indicator of intracellular signaling events, and any perturbation in intracellular signaling, however subtle, is reflected by changes in cellular morphology.

We have demonstrated that stimulation of GPCRs leads to certain morphological changes, which can be sensitively and precisely measured by cell–electrode impedance measurement. The major advantages offered by impedance measurement over traditional GPCR functional assays is that it can be used in cells expressing both exogenous or more importantly endogenous receptors. The preclusion of labels, reporters, and promiscuous G proteins allows for a more physiologic assessment of GPCR functions. Furthermore, the change in cell cytoskeleton or cell morphology that is measured by cell sensor impedance is an inherent cellular response that reflects the status of intracellular signaling. The kinetic element of the assay system allows for receptor-specific response profiles that can potentially be used to characterize orphan GPCRs. In summary, impedance readout allows another vantage point to assess GPCR functions and in combination with standard technologies and other emerging technologies such as BRET and FRET will undoubtedly add to our understanding of GPCR biology and drug discovery.

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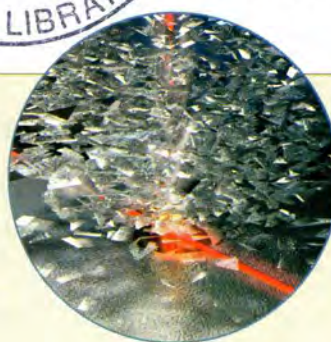
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Dual-Pulse
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**MS on the
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APPENDIX B

Monitoring electroporabilization in the plasma membrane of adherent mammalian cells

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ABSTRACT When an electrical potential of order one volt is induced across a cell membrane for a fraction of a second, temporary breakdown of ordinary membrane functions may occur. One result of such a breakdown is that molecules normally excluded by the membrane can now enter the cells. This phenomenon, generally referred to as electroporabilization, is known as electroporation when actual pores form in the membrane. This paper presents a unique approach to the measurement of pore formation and closure in anchored mammalian cells. The cells are cultured on small gold electrodes, and by constantly monitoring the impedance of the electrode with a low-amplitude AC signal, small changes in cell morphology, cell motion, and membrane resistance can be detected. Because the active electrode is small, the application of a few volts across the cell-covered electrode causes pore formation in the cell membrane. In addition, the heat transfer is very efficient, and the cells can be porated in their regular growth medium. By this method, the formation and resealing of pores due to applied electric fields can be followed in real time for anchorage-dependent cells.

INTRODUCTION

Electroporabilization is defined as the phenomenon of a normally selective biological membrane suddenly becoming permeable to a wide variety of ions and molecules under influence of a strong electric field. A special case of electroporabilization is electroporation, when small transient pores are formed in the membrane. Pore formation occurs when the transmembrane potential, which is normally about 60–110 mV, is raised to about 1 V (1–4). Under these conditions, the pores reseal soon after the high field is removed. If the transmembrane potential is raised further, irreversible membrane damage takes place, culminating in cell death (1, 2). Electroporabilization of cell membranes is commonly used to insert foreign material into the cell. Macromolecules, which are normally excluded by the plasma membrane, can be induced to enter the cells through the membrane pores (1–3, 5). Recently, however, some speculation has been made concerning whether large molecules like DNA enter cells through pores, or by endocytosis induced by the electrical field (5).

Electroporabilization has been studied in several ways, including rapid-freezing electron microscopy (6), detection of macromolecule entry into the cells (3, 5), and a relaxation in the membrane resistance (7–11). The last method is probably the one most suited for investigating the mechanism of pore formation and resealing, because the cells studied this way are not exposed to foreign material which might influence the results. Relaxation of membrane resistance has been measured by patch clamping (7, 8), fluorescent imaging (9) and current–voltage measurements (10, 11). In most cases reported in the literature, current–voltage measurements have been conducted on suspended cells placed between two closely spaced electrodes in a specially constructed “electroporation” chamber (10, 11). In such studies, a high voltage (10^2 – 10^3 V) was applied for a

very short duration (10^{-5} – 10^{-4} sec), and electroporabilization was detected as a change in conductivity (10) or a deformation of the applied square wave pulse (11). Such high voltages were necessary in order to cause the required potential drop across the cell membrane in the cell suspension, where the two electrodes were placed a few millimeters away from each other (10). The use of such high voltages caused unwanted Joule heating (4), and therefore required the use of special high resistivity solutions. It has been suggested that some mammalian cells are “unstable” in very high resistivity media (12). A different approach is to use cells attached to a substrate where they are in a monolayer. In the few reported cases of electroporabilization using attached cells (13, 14), however, the electrode geometry used was such that the main voltage drop occurred across the bulk of the electrolyte and not the cell layer. In these situations high voltage, and consequently, high resistivity media were required.

In this paper we report the use of current–voltage measurements to detect pore formation and monitor pore resealing in the plasma membrane of adherent mammalian fibroblastic cells. The method for measuring cell impedance used in this work was developed by Giaever and Keese, and has been explained in greater detail elsewhere (15, 16). The same system has now been used for electroporabilization experiments. The cells are grown on small gold electrodes (area $\sim 10^{-3}$ cm²) evaporated on the bottom of tissue culture dishes. A small alternating current (1 μ A) at a frequency of 4,000 Hz is passed between this electrode and a counter electrode (area ~ 2 cm²) placed some distance away (see Fig. 1 for circuit details). The electrolyte used is the regular tissue culture medium, which has a resistivity of ~ 54 Ω -cm. The potential drop across the small electrode is much larger than the potential drop across the solution or the large electrode; therefore, the impedance of the small electrode dominates the system (17). As cell membranes have a very high impedance, the presence of cells on this

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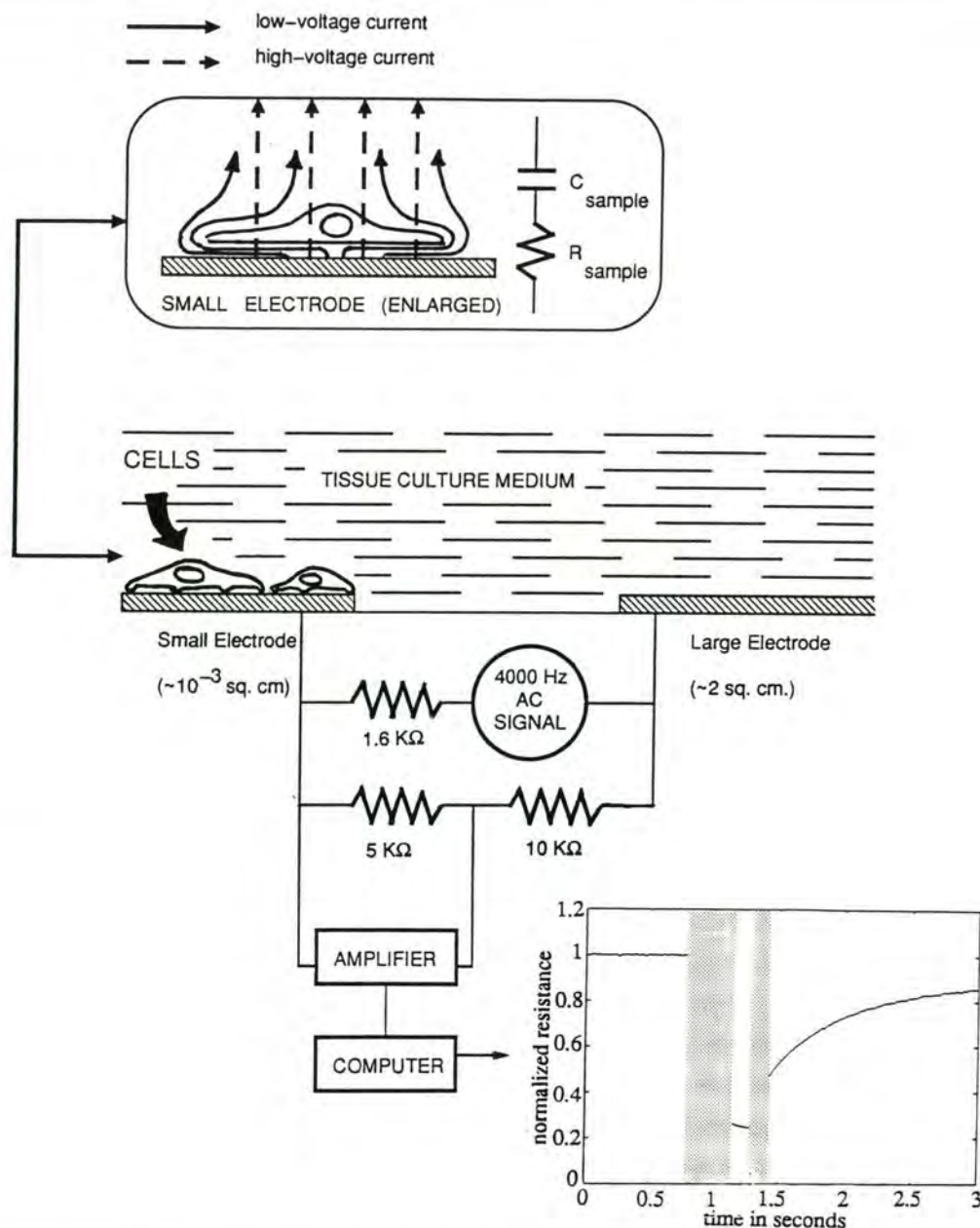


FIGURE 1 The basic setup used in the experiments. The voltage across the sample is too large to be measured by the amplifier; therefore, a voltage divider is used, and the amplifier measures only the voltage across the 5 K Ω resistor. This must be taken into consideration whenever the voltage across the sample itself is to be calculated—this voltage is roughly three times the measured voltage. The sample is treated as a simple RC series circuit, as shown in the inset. The position of the large electrode is not important because the impedance is dominated by the small electrode.

electrode greatly affects the measured impedance, and the resistance may go up by a factor of 4–8 for a confluent cell layer. Under these circumstances, any small change in impedance of the cell layer, either due to cell motion, morphological changes, or a change in membrane resistance, is easily detected. As will be shown later in this report, a 200 ms, low voltage (1 V–5 V) pulse will be sufficient to permeabilize the cell membrane. Because the Joule heating is constrained to a small volume close to the electrode, it can be shown that the rate of heat dissipation is sufficient to keep the temperature under control even with such low resistivity media. Pores in the

cells are detected as a sudden drop in the impedance of the cell layer on the electrode, which can be constantly monitored in this setup using a much lower applied voltage (~ 1 mV). The lower voltage mode has been found to be non-invasive (18), hence the cell layer can be monitored for long periods of time with no additional damage to the cells under study.

MATERIALS AND METHODS

Cells and cell culture. Transformed human lung fibroblasts WI-38/VA-13 (referred hereafter as VA13 cells), were used in all experiments. The cells were cultured in Dulbecco's Modified Eagle Medium

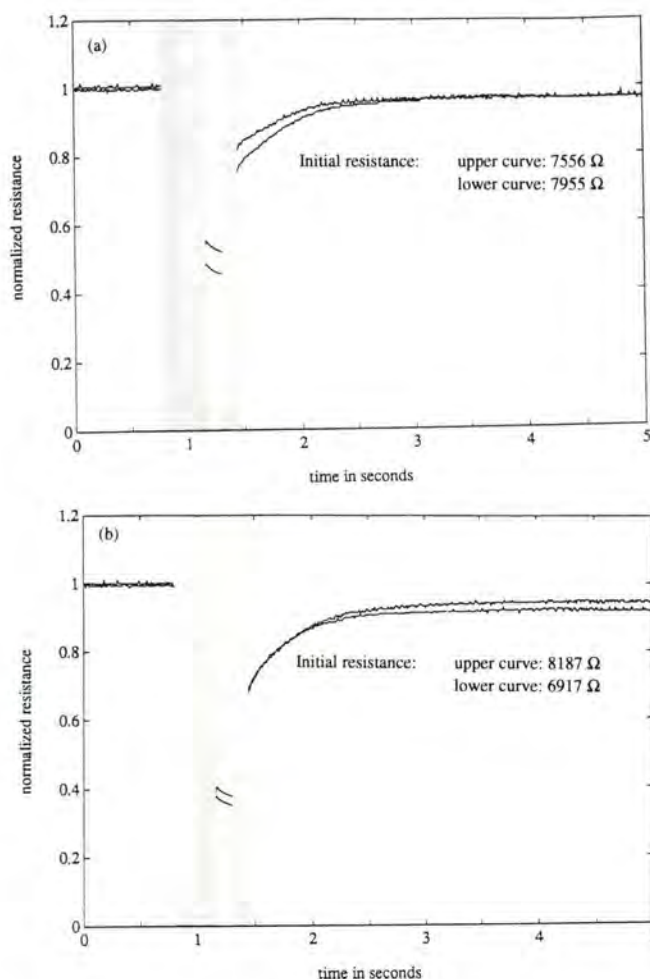


FIGURE 3 (a) Two different electrodes have been pulsed with a 2 V, 200 ms pulse each, and the responses of the two cell layers have been traced on the same plot. The upper curve represents a cell layer with a resistance ratio (ratio of resistance of the cell-covered electrode to that of the empty electrode) 5.00, while the lower curve represents a cell layer with a resistance ratio 6.00. Both these electrodes are of comparable surface area. (b) This shows the results of plotting two electrodes with very different surface areas but the same resistance ratio (6.1), and each pulsed with a 3 V, 200 ms pulse.

that a change in Z_n due to the field cannot explain the large resistance changes during pulsation.

To look at the impedance changes due to the other factors in more detail we shall use a model developed by Giaever and Keese (20). According to this model, at 4,000 Hz, the current from the electrode normally flows in spaces under the cells and between the cells, with only a small capacitively coupled current passing through the intact plasma membrane (see inset in Fig. 1 for details). Only if the membrane is punctured will a large amount of current flow through the cell itself. Eq. 2, based on this model, relates the specific impedance of the cell-covered electrode, Z_c ($\Omega\text{-cm}^2$), to the specific impedance of the empty electrode, Z_n ($\Omega\text{-cm}^2$), the specific impedance of the membrane itself Z_m ($\Omega\text{-cm}^2$), the specific resistivity between the cells, R_b ($\Omega\text{-cm}^2$), and a parameter α =

$r_c(\rho/h)^{0.5}$ ($\Omega^{1/2}\text{-cm}$), where r_c is the radius of the disk-shaped cell, ρ is the resistivity of the electrolyte, and h is the height of the space between the ventral side of the cell and the substratum:

$$\frac{1}{Z_c} = \frac{1}{Z_n} \times \left(\frac{Z_n}{Z_n + Z_m} + \frac{\frac{Z_m}{Z_n + Z_m}}{\frac{\gamma r_c}{2} \frac{I_0(\gamma r_c)}{I_1(\gamma r_c)} + R_b \left(\frac{1}{Z_n} + \frac{1}{Z_m} \right)} \right). \quad (2)$$

Here, I_0 and I_1 are the modified Bessel functions of the first kind of order 0 and 1, respectively, and

$$\gamma r_c = \alpha [(1/Z_n) + (1/Z_m)]^{0.5}. \quad (3)$$

For the VA13 cells under consideration, the standard values found are $R_b = 2.2 \Omega\text{-cm}^2$ and $\alpha = 7$ (r_c has been measured at $11 \times 10^{-4} \text{ cm}$) [20]. The normal membrane resistivity of an intact membrane is of the order of $10^3 \Omega\text{-cm}^2$ while the capacitance of the membrane is of the order of $10^{-6} \text{ Farads/cm}^2$. Eq. 2 shows that Z_c , the parameter actually measured, will change if either of Z_m , Z_n , α , or R_b changes. A change in Z_n can be brought about only by an electrochemical reaction of the small gold electrode in contact with the electrolyte which, as we have already seen, is negligible compared to the other changes. Cellular motion, or a change in cell morphology, can result in either a change in α , or a change in R_b , whereas membrane permeability will cause a change in Z_m .

When a cell layer is shocked with an applied electric pulse, the cells may respond by movement or a change in morphology (21, 22), and these would alter both α and R_b . If only α and R_b were to change, a very large decrease in both these parameters, arising from major alterations in cell morphology, would be required to explain the results. However, no large-scale change in cellular morphology has been observed by simultaneous time-lapse video-micrography in the 5-second time ranges recorded in Fig. 2. In addition, any change in impedance due to cellular motion or morphological changes would be much slower than the changes seen. Even if the cells could break away from the electrode in the span of milliseconds due to the applied field, a fast recovery seems unlikely, since even small movements normally require several minutes (23).

The explanation, therefore, is that the membrane impedance, Z_m , changed drastically during the pulse, accounting for the large drop in impedance observed in Fig. 2. By using Eq. 2 it is possible to calculate the change in Z_m necessary to explain the observed resistance changes. In Fig. 5, we have used this equation to plot changes in electrode resistance against corresponding changes in membrane resistivity. The width of the curve represents variations in parameters such as electrode area, α and R_b , as the actual resistance of the cell-covered

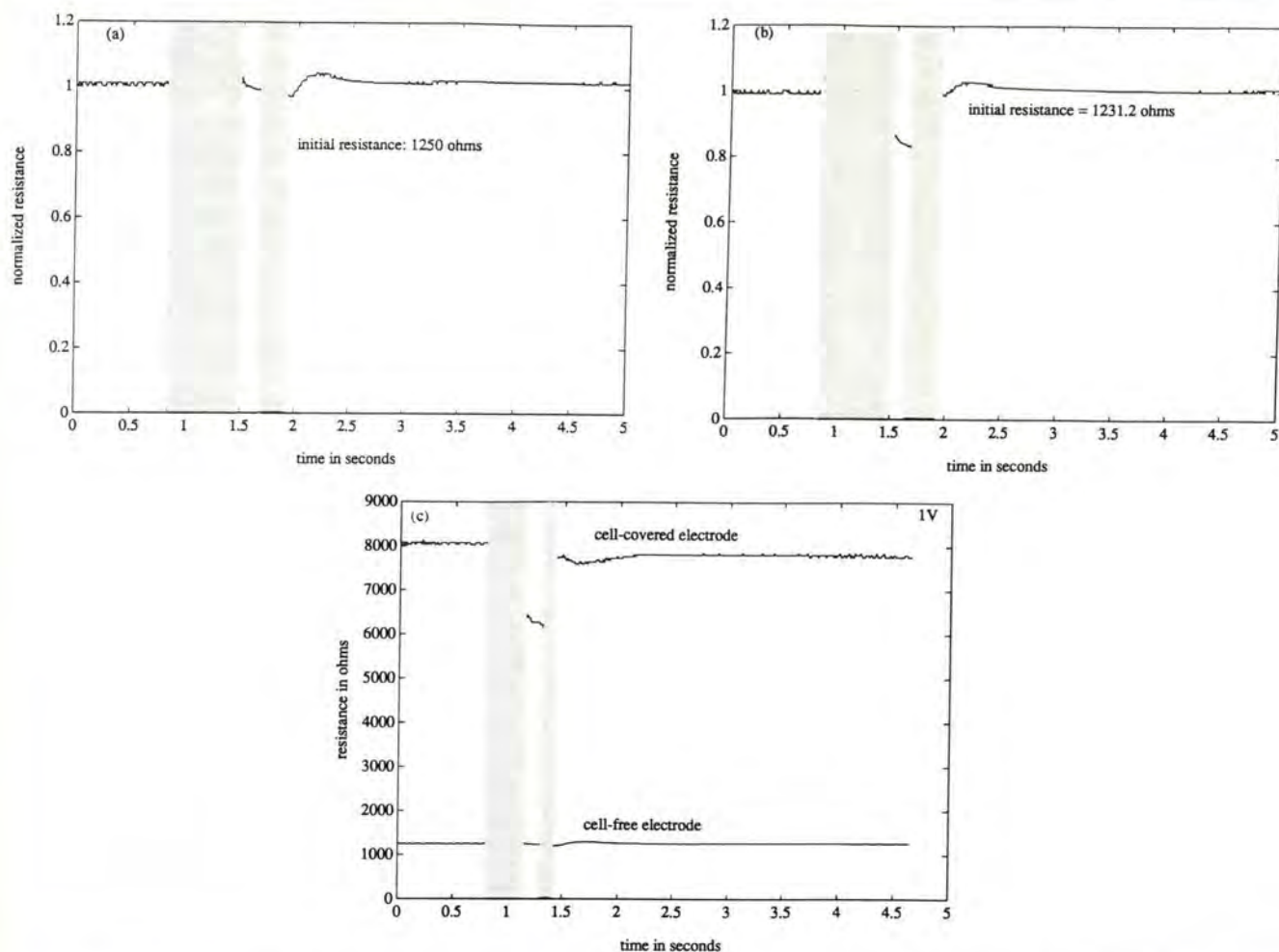


FIGURE 4 Observed changes in the measured resistance of a cell-free electrode due to application of (a) 1 V and (b) 2 V. We have not shown higher voltages because such high voltages never appear across the electrode when it is covered with cells. In (b) the resistance falls slightly during the pulse. This is due to the non-ohmic behavior of the gold electrode in contact with the electrolyte. In (c), we show the results from Figs. 2a and 4a on the same scale (not normalized). It is clear from this figure that compared to the contribution of the cells on the resistance changes, the contribution of the electrode is negligible. The effect of only the cells can be obtained by subtracting out the lower curve from the upper one.

electrode would depend on these as well. We can use this figure to calculate the fall in membrane resistivity that would cause the measured drop in resistance. As an ex-

TABLE 1 Response of cells to pulsation

Applied voltage across cell-covered electrode	Intensity of dye	Percentage of cells stained as seen on the electrode
0 volts	—	0%
1 volt	—	0%
2 volts	—	0%
3 volts	+	<20%
4 volts	++	>50%
5 volts	+++	>50%

Increase in number of cells and intensity of blue stain inside cells with increased pulsing voltage, as measured by eye estimation from photographs of the blue-stained cells taken soon after they had been pulsed in the presence of the enzyme horseradish peroxidase. No blue stained cells were seen upon application of 0–2 volts, after which the amount of stain on each electrode increased rapidly.

ample, we shall consider the steep resistance drop shown in Fig. 2b, where 2 V was applied to the electrode. Using Eq. 1, we can show that this is equivalent to 1.3 V across the cell layer or about 0.65 V across each membrane. We have considered the entire voltage drop to be across the basal and apical membranes of the cells because the cytoplasm in between has very low resistivity. From Fig. 2b we can see that the resistance dropped to 55% of its original value during the high voltage pulse. Using Fig. 5, we can estimate that the membrane resistivity, R_m , has dropped from about $1,000 \Omega\text{-cm}^2$ to about $2 \Omega\text{-cm}^2$ as a result of the pulse. Such a large change in membrane resistivity can only be explained by pore formation.

Although our system does not allow us to distinguish between a small population of large pores from a large population of small pores, the measurement of the drop in membrane resistivity as obtained from Fig. 5 allows us to estimate the average diameter of the pores formed, if we make an assumption about the number of pores

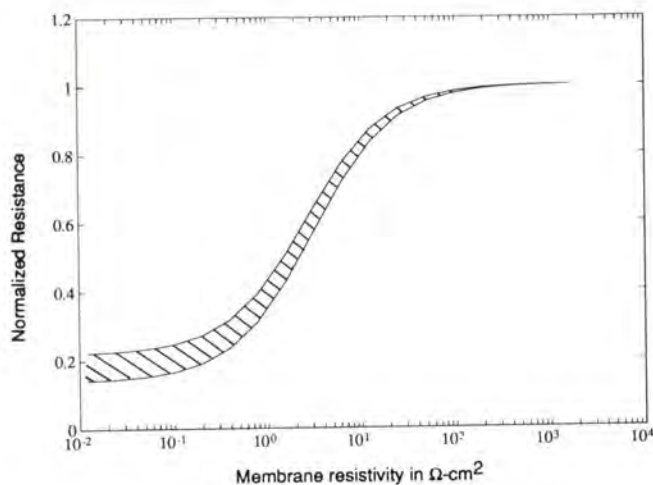


FIGURE 5 The theoretical relationship between the cell membrane resistivity and the normalized electrode resistance obtained using Eq. 2. To first order, no change in the membrane capacitance occurs with the formation of pores. The width of the curve represents variations between the electrodes in parameters such as electrode area, α and R_b (see text for details).

formed per cell. This estimate will give us an idea of the amount of damage we are doing to the cell membrane and may be useful in controlling the factors affecting the cell response. If we assume n pores of fixed size per cm^2 on each side of the cell layer, the pore radius can be calculated from the relationship:

$$R_m = [\rho t_m / (\pi r_p^2) + \rho / (2r_p)] \times 2/n \quad (4)$$

where ρ ($54 \Omega\text{-cm}^2$) is the resistivity of the tissue culture medium, t_m (8 nm) the membrane thickness, and r_p is the pore radius. The first term in the expression is the resistance of a single pore, and the second term is the sum of the constriction resistance on each side of that pore (17). The factor of 2 outside the bracket is due to the fact that the current goes through two membranes. If $n = 1$, the pore diameter would be roughly 1,000 nm for an application of a 2 volt pulse, and if $n = 1,000$, the diameter would be 3 nm. Since the enzyme horseradish peroxidase (diameter 5.34 nm) did not enter the cells at this voltage, the latter seems more probable. Data presented by others on erythrocytes (6, 24) also suggest that for cells of the size used, a thousand pores per cell would be a reasonable estimate. For example, this number is in agreement with the observations of Chang and Reese (6), who have found less than seven pores per μm^2 , which, coupled with the fact that the radius of the cells we use is $11 \mu\text{m}$, and therefore a cell area of about $380 \mu\text{m}^2$, gives approximately 2,000 pores per cell. This number also agrees with the estimates of Dimitrov and Sowers (24), but exceeds somewhat the estimate of Kinoshita and Tsong (3).

The observations made in Fig. 2 can now be explained in the light of the above discussion. When a voltage pulse

is applied, the permeability of the membrane increases, because of pore formation. These pores start resealing as soon as the high voltage is turned off, and therefore, recovery of initial membrane resistance is observed. This is in accordance with the observations of others (6, 9, 10) who have determined that the pores form within $100 \mu\text{s}$ after the application of the pulse and start resealing within a few milliseconds. It has been proposed that pore resealing takes place in three stages (25)—a rapid initial decrease in pore size, a slower secondary decrease, and a complete resealing over a long period of time. The data presented in Fig. 2 are in accordance with these observations—we see that the slope of the resistance decreases over time. It is significant that although the drop in resistance during the pulse is proportional to the magnitude of the pulse, at the end of the experiment in all four cases, the resistance is 96% of the initial resistance. This may mean that the VA13 cells used have a characteristic resealing time, independent of the magnitude of the pulse, unless a threshold is crossed, when irreversible membrane rupture takes place. More detailed studies will show whether the 4% drop in resistance seen at the end of the experiments presented in Fig. 2 results from the presence of very small pores, or changes in cell morphology. It may be noted that data reported in the literature have found the time taken for complete resealing of the pores to vary widely—from a few seconds to a few hours (25).

It has been observed that the response of the cells to pulsation varied with the degree of confluency of the cell layer on the electrode. This is demonstrated by the results reported in Fig. 3. In Fig. 3a we have plotted the response of two different cell layers, both pulsed with 2 volt, 200 millisecond pulses. The upper curve represents a cell layer of resistance ratio 5.00, while the lower curve represents a cell layer of resistance ratio 6.00. Using Eq. 1, and taking into consideration both the resistive and reactive parts of the impedance of the cell-covered and cell-free electrode, the potential across the cell layer on application of the pulse is found to be about 1.1 V in the first case, while it is about 1.2 V for the second. The difference in voltage caused a slight difference in the response of the cell layer even though the voltage applied across the cell-covered electrode in both the cases is the same. If the degree of confluency of two cell layers is the same, their response to pulsation is similar. This is illustrated in Fig. 3b, where two electrodes of widely different surface areas were used, but the resistance ratio in both cases was approximately the same. Both electrodes were pulsed with 3 volt, 200 millisecond pulses, and in this case, the potential across the cell layer was similar. The slight difference in cell recovery may be due to the fact that while the cell layer represented by the upper curve was two days old, that represented by the lower curve was only one day old. The difference in cell response according to the age of the cell layer has also been observed by Raptis and Firth (14).

For clarity, we have only talked about the measured changes in resistance due to pore formation. As is evident from Eq. 2, very similar changes in the measured reactance take place due to formation of pores. During these experiments, we have tracked the changes in reactance as well, and these measurements support and confirm the conclusions drawn in this paper. Finally, the method can be improved by using an amplifier that has a faster response time and can take data more frequently. This will allow us to look at further details in the formation and resealing of the membrane pores.

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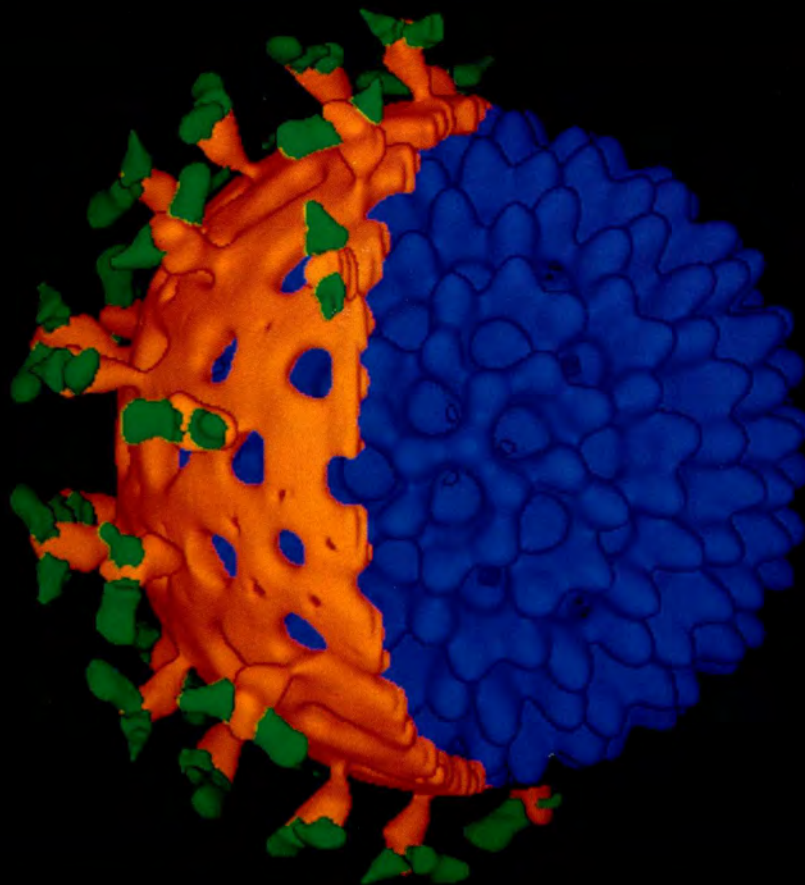
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APPENDIX C

Performance of a thin film microelectrode array for monitoring electrogenic cells in vitro

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Abstract

A planar array of microelectrodes has been developed for monitoring the electrical activity of electrogenic cells in a cell culture by an extracellular recording mode. The design, the fabrication technique, and the characterization of the array by impedance measurements is described. The device is applied to monitor extracellular signals produced by cultured chicken embryo cardiac myocytes.

Keywords: Microelectrode array; Extracellular recording; Cell culture; Cardiac cells

1. Introduction

Monitoring signals of electrogenic cells in vitro over extended periods of time is of principal interest, e.g. for investigations of neuronal networks and their changes in function, as they may be induced by external (chemical, physical) and internal parameters [1]. A particularly suitable technique for monitoring simultaneously the activity of several electrically active cells is to record extracellularly spike activity with a planar microelectrode array integrated into the bottom of a tissue culture chamber. Several different designs of microelectrode arrays have been developed in this context [1–7]. This paper describes a new design and the application of such a thin film microelectrode array. It was produced by conventional semiconductor fabrication techniques. The microelectrodes were characterized by impedance measurements, and the spatial variation of the electrical response characteristics of the platinized microelectrodes was evaluated in detail. Finally, results from extracellular recordings are described. The electrical signals were produced by cultured aggregates of cardiac myocytes of chicken embryo.

2. Design and fabrication

The device consists of an array of 60 gold microelectrodes, covered with platinum black, on a glass substrate. The circular electrodes have a diameter of 10 μm . They are arranged in an 8 $\times$ 8 matrix (missing electrodes at the corners) with a distance of 100 μm from tip to tip (Fig. 1b). The insulation layer is made of silicon nitride. The recording area of the microelectrodes is enclosed in a glass ring which forms the chamber for the cell culture (Fig. 1a). The thin film microelectrode arrays were produced by conventional semiconductor fabrication processes. Pyrex glass (Corning 7059, 2 $\times$ 2 inches) was used as a substrate, and gold was applied as an electrode material. To improve the adhesion of the 300 nm gold coating, a layer of 10 nm Cr was deposited first. The gold was patterned using standard photoresist (Shipley S1400/31) and was etched in a solution of I/KI. The Cr was etched in 1% HCl solution. This resulted in a pattern of gold conductors extending from connector pads at the edge of the glass plate to the electrode matrix at the centre. After removal of the photoresist (Shipley Remover A140), the insulation layer of Si₃N₄ (500 nm) was evaporated onto the whole surface of the substrate by a PECVD-process. Reactive ion etching (RIE) was used to pattern the insulation layer and to create openings above the tips of the conductor and for the connector pads. The arrays were

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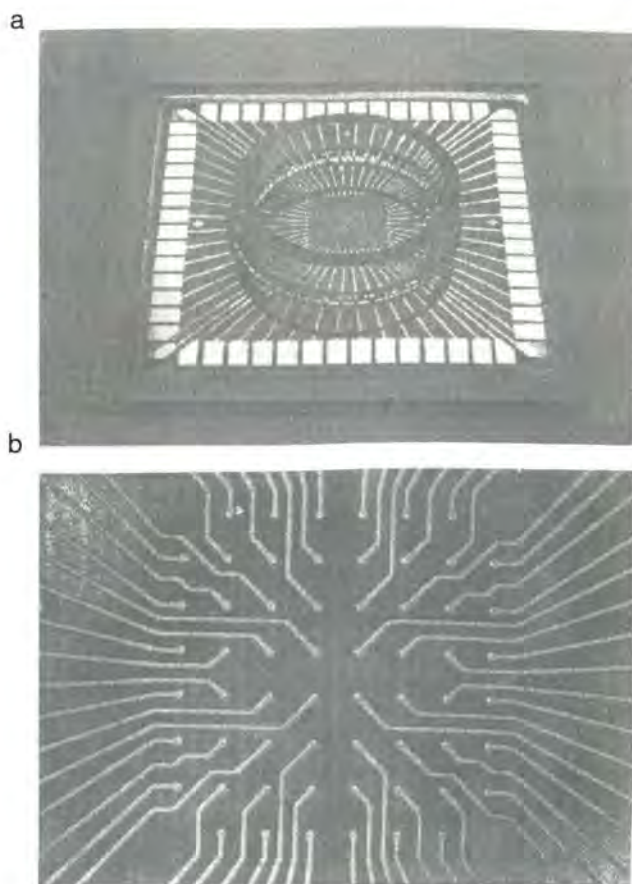


Fig. 1. The microelectrode array: the complete recording device (a) and the recording area on a finer scale (b).

O₂-plasma-etched to produce a hydrophilic surface. For details of the experimental setup, see [5,6].

3. Impedance measurements and platinization

To improve and homogenize the impedances Z of the microelectrodes, they were platinized subsequently. Impedance measurements were performed with a Hewlett Packard 4284 precision LCR meter at a constant frequency of 1 kHz and a 100 mV signal amplitude. A programmable multiplex unit connected to the array holder switched each one of the 60 microelectrodes to the LCR meter. For impedance and simulation measurements, we used 0.90% NaCl solution (physiological saline) at 22°C. Kohlrausch's solution (3% PtCl₂, 0.025% lead acetate) was used for platinization. Before the deposition of the platinum, typical values of $|Z|$ at 1 kHz were 4 MΩ. By electroplating of platinum, these values were adjusted to 400 kΩ (± 10 kΩ). Impedances around 400 kΩ appeared to be the best compromise between the required low electrode impedance for high signal/noise recording and the stability under bioassay conditions of the platinum layer formed on the electrodes.

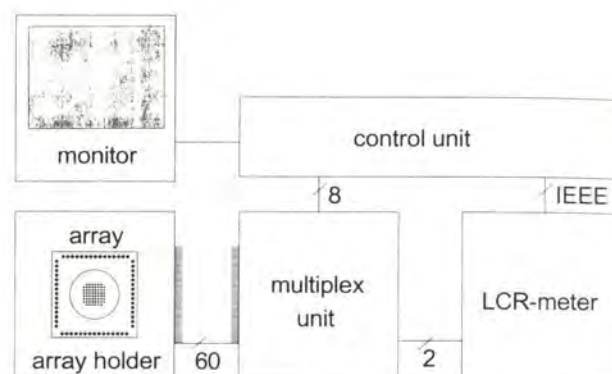


Fig. 2. Experimental setup for measuring the spatial sensitivity of the microelectrodes.

4. Simulation measurements

The influence of the geometric arrangement between cell and electrode on the extracellular recording was simulated by a spatial variation of externally applied local electric fields. For this purpose, a sinusoidal signal (1 kHz) was applied across the barrels of a double-barrelled glass micropipette (theta-style) to mimic the action potential of the cell (Fig. 2). An x,y,z -piezo actuator with a maximum lateral shift of 100 μm moved the micropipette over a single microelectrode. The response characteristics of the electrode was measured as a function of the vertical and lateral distance between this micropipette tip and the microelectrode in saline solution. The detected signal amplitude reached the noise level at a vertical and lateral distance of around 20 μm between micropipette tip and microelectrode (Fig. 3). Fig. 3 illustrates that cells which are electrically detected with one microelectrode cannot be detected at the neighbouring electrode unless parts of the cell cover both electrodes.

To maximize the amplitude of a detected extracellular

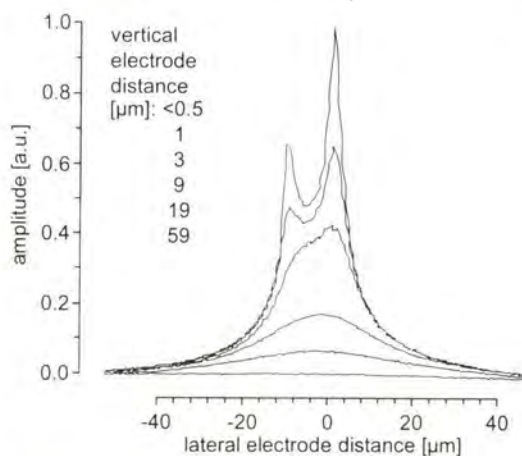


Fig. 3. Amplitude of the detected signal as a function of the vertical distance between micropipette tip and microelectrode. For details, see text.

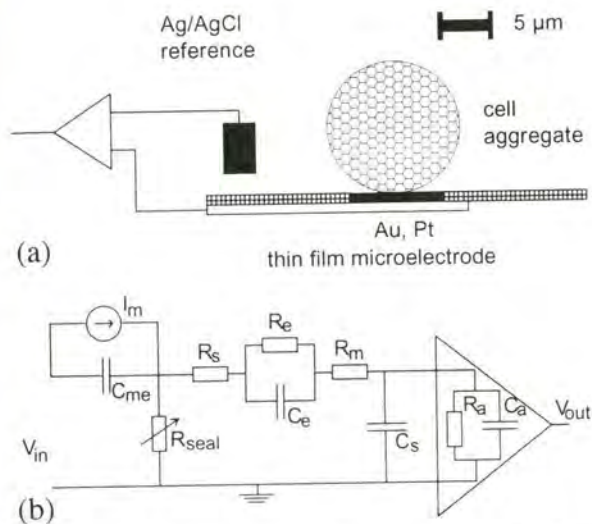


Fig. 4. Experimental setup for extracellular recording with the microelectrode array. (a) the extracellular recording device and (b) the electronic circuit diagram.

potential, the cell should evidently be positioned as close as possible to the microelectrode. In the future this will be optimized even better by the careful choice of cells, substrates, and, in particular, by arranging adhesion molecules between both. The goal of this effort is to achieve a lateral resolution which leads to additional analytical information only if the attachment of the cells to the electrodes has comparable extracellular impedance values between the membrane and the electrode.

5. Extracellular recording

The experimental setup for extracellular recordings on cell ensembles is depicted in Fig. 4. As a model system, a cardiac cell culture was taken from 11-day chicken embryos (E11). This choice was motivated by four reasons: first, embryonal cells can be kept alive much easier than adult cells. Second, cardiac cells form aggregates which

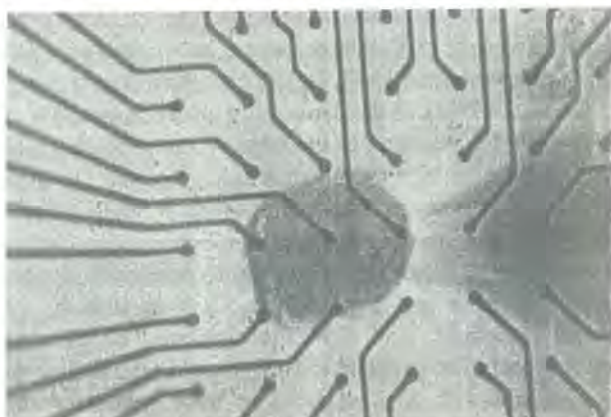


Fig. 5. Aggregate of heart muscle cells at the microelectrode array.

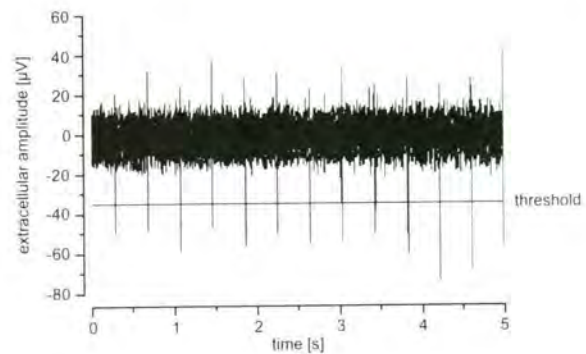


Fig. 6. Extracellular spikes of heart muscle cells measured with one electrode of the microelectrode array.

can be handled easily. Third, the whole aggregate generates a higher common action potential which can be monitored easily (about the nature of the action potential see, for example [8]). This aspect is of particular importance if one considers the comparatively small signal amplitudes of individual nerve cells [5]. The fourth reason is that the influence of drugs on heart cell activity continues to be an interesting topic for particular applications.

After 1 day of cultivation the dissociated heart muscle cells form aggregates if they are kept in suspension. Aggregates cultivated for 4 days were used for recording. In this stage of cultivation the aggregates consisted of up to 500 cells with aggregate diameters of up to 200 μm . For electrical recordings a suspension of the aggregates was transferred to the multi-electrode array. Adhesion of the aggregates was complete within about 1–2 h of incubation (Fig. 5). The extracellular recordings were performed at 37°C in a solution which contained (in mM): 136.9 NaCl, 2.68 KCl, 1.84 CaCl_2 , 1.03 MgCl_2 , 5.5 D-glucose, and 10 HEPES. The pH was adjusted to 7.3. A single pulse shows a peak to peak amplitude of about 120 μV (at the electrode where it is attached best) and a duration of about 1 ms. Interspike intervals are in the order of 0.4 s.

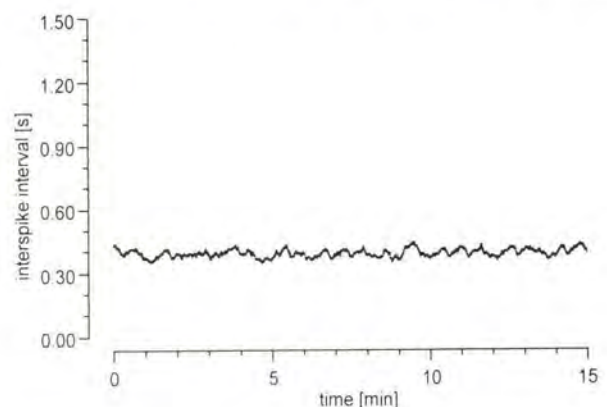


Fig. 7. Long term control measurement of the interspike interval of the heart muscle cells.

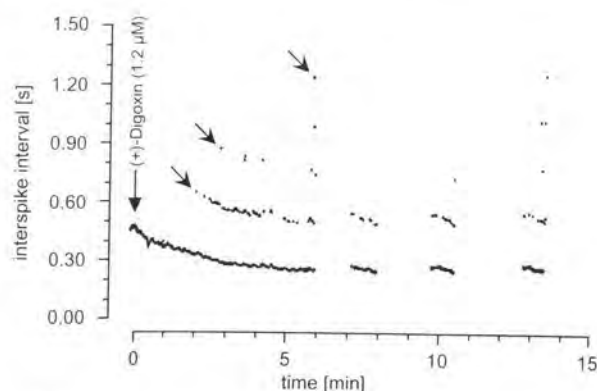


Fig. 8. Interspike interval of heart muscle cells with a heart stimulating drug.

The spike pattern of the aggregate was fairly constant with an average frequency of about 2.5 Hz (Fig. 6).

In order to estimate the influence of heart active drugs we had to gather knowledge about the long time behaviour of the cardiac cell aggregate. We achieved this by introducing a threshold into the measurement to reduce the amount of 200 kB/s raw data. Due to this threshold we only recorded spikes of an amplitude greater than $37 \mu\text{V}$. This leads to an extension of the time scale up to 15 min. For this time interval in Figs. 7 and 8 the variation of the time distance between two spikes is shown.

In Fig. 8 the effect of the heart-active drug digoxin on the spike pattern is presented. Digoxin, a blocker of $\text{Na}^+/\text{Ca}^{2+}\text{ATPase}$, gives rise to a continuous increase of the spike activity, i.e. the interspike interval is decreased. After 6 min the increase of spike activity is blocked completely for a period of about 1 min. Previous to the activity block some spikes skipped as indicated by the spots representing multiple spike intervals (marked by arrows). This arrhythmic behaviour is most prominent immediately before the activity blocks occur.

6. Conclusion

The present approach to record electrical signals by multi-electrode arrays proves to be a useful tool to monitor and analyze drug effects on electrical activity from cultivated cardiac cells.

Acknowledgements

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Biographies

Wolfgang Göpel received his Ph.D. from the University of Hannover in 1971. After visiting scientist positions at the Xerox Palo Alto (CA), Xerox Webster (NY) and IBM Watson (NY) Research Centers he was appointed as Full Professor of Physics at the Center of Surface and Submicron Analysis (Bozeman, MT, USA). Since 1983 he has been the Department Head of the Institute of Physical and Theoretical Chemistry and the Director of the Center of Interface Analysis and Sensors at the University of Tübingen. Main research interests (370 original papers) include interface properties of new materials for chemical sensors, catalysts and microelectronic, molecular electronic and bioelectronic devices.

Armin Mohr graduated in 1995 in biophysics at the University of Tübingen. Most of his work was done at the Natural and Medical Science Institute. His work includes the signal transduction of electroactive cells like tectum opticum cells, nerve cells and cardiac cells. Furthermore he built up a microelectrode array for extracellular recording.

Hugo Hämmerle graduated in biology in 1982 at the University of Holhenheim and received his Ph.D. in 1985 at the University of Tübingen, where he worked as a scientific assistant until 1987. In 1987 he moved as a group leader to the NMI at the University of Tübingen. From 1990–1995 he was head of the Department of Biology. In 1995 he became Vice President and head of the Division of Life Sciences at the Natural and Medical Science Institute (NMI). His research includes multichannel recording of neural network activity with 60 microelectrode arrays, growth changes and intracellular Ca^{2+} oscillations of diploid osteoblasts and fibroblasts induced by extreme low frequent electromagnetic fields and establishment of a co-culture with lung cells as *in vitro* model of the blood–air barrier.

Wolfgang Finger is electrophysiologist and graduated

at the University of Mainz in 1974. At the Department of Physiology of the Technische Universität München he received his Ph.D. in 1979, habilitated in 1984, and became a lecturer for physiology in 1985. In 1988 he moved to the NMI at the University of Tübingen and built up the Department of Biophysics. He is now head of this department and his research mainly includes signal transduction processes in among nerve cells.

Karl Josef Föhr graduated in biology in 1986 and received his Ph.D. in 1990 at the University of Ulm. In 1991 he moved as research assistant to the Department of Biophysics at the NMI, a research institute associated with the University of Tübingen. His research mainly

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Wilfried Nisch graduated in physics in 1982 at the University of Tübingen and received his Ph.D. in 1987 at the Institute of Applied Physics, University of Tübingen. In 1987 he became a group leader to the NMI at the University of Tübingen. Since 1990 he has been head of the Department of Applied Physics. His research includes thin film technology, microstructure technology and analysis, electron microscopy, physical electronics and bioelectronics.

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B **CHEMICAL**

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APPENDIX D

Portable cell-based biosensor system for toxin detection

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Abstract

A portable cell-based biosensor has been developed and characterized. The prototype system relies on extracellular recording from excitable cells cultured over an array of platinized gold microelectrodes. Extracellular potentials were bandpass filtered between 80 Hz to 2.8 kHz and amplified with a selectable gain of either 1000 or 5000. The input-referred noise level of the system was only 8.7 μV_{RMS} in the laboratory setting, reaching only 10.6 μV_{RMS} in an outdoor environment, more than sufficient for measurement of extracellular potentials from excitable cells. The system also incorporates a feedback control system for temperature regulation and a 36-channel multiplexer for selection of up to four output channels for simultaneous display. Wherever possible, low-cost 'off-the-shelf' components were utilized in this prototype biosensor design. Using this system, extracellular recordings from chick myocardiocytes were performed under both laboratory and outdoor conditions. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Amplifier; Biosensor; Cardiac myocytes; Extracellular recording; Portable

1. Introduction

Portable biosensors utilizing immobilized antibodies have been developed for environmental detection of biological analytes [1,2]. While immunosensors provide a means for identification of a biological threat or toxin, binding assays cannot offer insight into the functional consequences of an analyte–tissue interaction. To address the need for physiologically relevant, function-based assays, several groups have explored the utility of tissues [3] and cells [4,5] as sensor elements. One means of acquiring cellular functional information for biosensor applications involves extracellular recording from excitable cells [6–8]. Our long-term goal is the development of a portable cell-based biosensor for detection of an array of chemical/biological agents. The purpose of this paper is to describe and characterize a prototype extracellular recording system suitable for field use. The major hardware features of this modular

system include a planar array with 36 microelectrodes and supporting hardware incorporating AC-coupled buffer and filter stages constructed from 'off-the-shelf' components. To demonstrate the utility of this system, extracellular recordings from chick cardiac myocytes have been performed.

2. Methods

2.1. Microelectrode array fabrication and platinization

Planar microelectrode arrays were designed and fabricated at the Center for Integrated Systems at Stanford University. Each array has 36 microelectrode sites that are 10 μm in diameter (Fig. 1). For array fabrication, a (100) orientation, p-type silicon wafer was thermally oxidized to provide an insulating 500 nm SiO_2 layer. Gold electrodes, interconnecting traces and bond pads were patterned using a bilayer lift-off structure consisting of sputter-deposited silicon (500 nm) and Shipley 1813 photoresist (1 μm). The photoresist was patterned

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using standard photolithographic techniques followed by an isotropic silicon plasma etch (CF_4/O_2) to undercut the sputtered silicon layer. A 500 nm layer of gold was then sputter-deposited using chromium (25 nm) as an adhesion layer, resulting in a Cr/Au bilayer on the exposed SiO_2 surfaces and on top of the photoresist. Acetone was used to dissolve the photoresist and thereby remove the Cr/Au bilayer not adhering to the SiO_2 substrate. The remaining sputtered silicon was then plasma stripped ($\text{C}_2\text{ClF}_5/\text{SF}_6$) to leave the desired gold microelectrodes, interconnecting traces and bond pads. To provide chemical and electrical insulation, the interconnecting traces and electrodes were then passivated with 1 μm of plasma-enhanced chemical vapor-deposited silicon nitride; this process was performed at low temperature (300°C) to limit gold roughening. A mixed frequency process (rapidly cycling between high and low frequencies) was utilized to allow sub-monolayers of tensile and compressively stressed silicon nitride to be deposited, resulting in overall near zero-stress films. Following passivation, windows were cut through the film using an anisotropic plasma etch ($\text{C}_2\text{ClF}_5/\text{SF}_6$) to define the electrode geometries and open the bond pads. The wafer was then diced and cleaned. Arrays were mounted in a ceramic 40-pin dual inline package (DIP; Spectrum Semiconductor Materials, San Jose CA) and bond-wire connections were made between the die and DIP. The bond-wires were encapsulated in a non-cytotoxic epoxy suitable for insulation (EpoTek 87GT, Epoxy Technology, Billerica, MA). For use in our cell culture facility, 35 mm polystyrene culture dish with a hole drilled in the center were adhered with the epoxy to the microelectrode arrays.

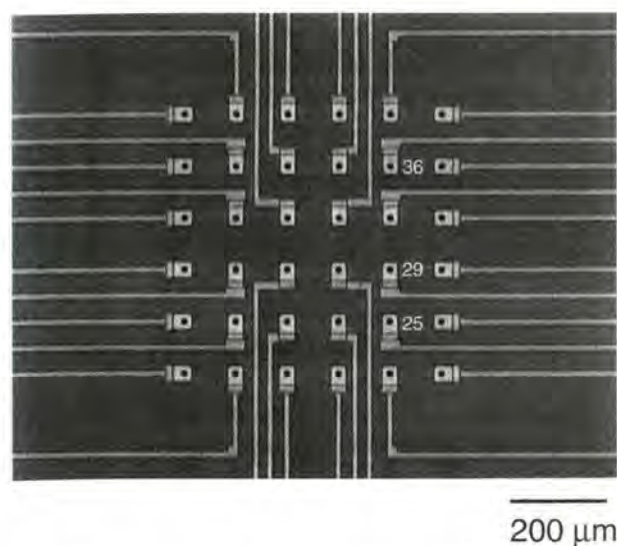


Fig. 1. Planar microelectrode array detailing individual platinumized, 10 μm diameter recording microelectrode sites. Microelectrodes 25, 29, and 36 are identified as sites for the extracellular recording example in Fig. 5.

Microelectrodes were electroplated with platinum black to decrease impedance values for adequate recording of biopotentials. Electrode impedance was measured in 0.9% saline with a LCR meter (model 4284A, Hewlett Packard, Rockville, MD) with an applied signal of 5 mV_{RMS} . The microelectrode impedance at 1 kHz was ~ 1 to 3 $\text{M}\Omega$ prior to platinization. To deposit platinum black, 1 V dc was applied at the microelectrode site for 30 s in a solution containing 5% chloroplatinic acid and 1% lead acetate (YSI, Yellow Springs, CO) where the current was limited to 5 nA μm^{-2} . After electroplating, the microelectrode impedance decreased to less than 300 $\text{k}\Omega$ at 1 kHz.

2.2. Data acquisition

In the cell experiments, the system output was recorded using a digital audio tape recorder (model RD-135T, TEAC, Montebello, CA). The RD-135T has a preamplifier stage with its gain set to maximize the 14-bit A/D conversion with a full scale input voltage range of ± 2 V yielding a sampling error of ± 244 μV with a sampling rate of 10 kHz per channel. For off-line analysis, the RD-135T output was digitized using a Digidata 1200B data acquisition board and Axotape version 2.0 (Axon Instruments, Foster City, CA) at a rate of 25 kHz and stored to the hard disk of an Intel Pentium-based PC.

2.3. System design considerations

The prototype biosensor was designed using 'off-the-shelf' components wherever possible. The biosensor is contained within an aluminum 'carrying case' 42 cm long, 23 cm wide, 21 cm high which weighs 12.4 kg with battery packs installed (Fig. 2A). The buffer and amplifier boards were designed to be stacked. The power supply, temperature control, and multiplexer are isolated from the buffer/amplifier stack by internally shielding each subsystem. For biosensor remote operation, three rechargeable nickel-cadmium (Ni-Cd) battery packs (model 1800_{MAX}, SR Batteries, Bellport, NY) were used, each delivering 18 V at a maximum current of 1.8 A. A standard 40-pin DIP containing the microelectrode array is held in a zero-insertion-force (ZIF) socket mounted on the buffer board (Fig. 2B). Between the ZIF and the DIP, a heating foil (model HK5221R13.0L12B, Minco, Minneapolis, MN) was installed to provide temperature stability which is a necessity for the cultured cells. A resistive heater controller (model RHM-4000, Wavelength Electronics, Bozeman, MT) featuring PID feedback and excellent stability was used to drive the thermal heating foil. Extracellular signals from the microelectrode array are ac-coupled to the followers (LMC6044, National Semiconductor, Santa Clara, CA) on the buffer stage (Fig. 3A). The ac

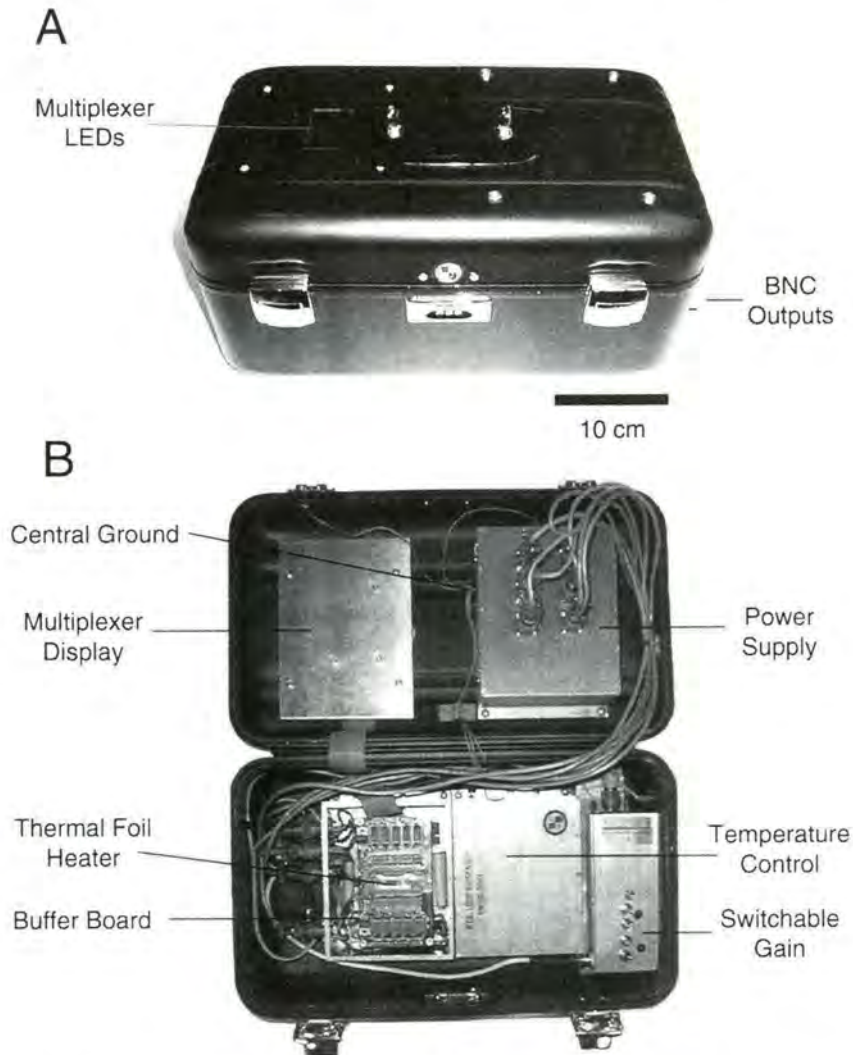


Fig. 2. Portable biosensor system. A, closed case view of the biosensor. B, Open case view showing several internal subsystems.

coupling eliminates dc signal components and also ensures that input offset currents cannot produce electrochemical changes at the cell–electrode interface. The shunt resistance of 45 M Ω (Dale Electronics Inc., Columbus, NE) was chosen to ensure that for signals at 1 kHz, there would be less than 1% attenuation of the extracellular signal amplitude. Each of the buffered channels is passed to an amplifier stage (LT1014, Linear Technology, Milpitas, CA) designed to yield high-pass and lowpass corner frequencies of 80 Hz and 2.8 kHz, respectively, and a gain of 200. The amplifier outputs are passed to a 36 channel multiplexer allowing the user to identify active microelectrode sites. A momentary contact rocker switch mounted on the top of the biosensor is buffered with Schmidt trigger (74HC14, Harris Semiconductor, Melbourne, FL) to drive two 4-bit up/down binary counters (DM54LS193, National Semiconductor), the binary output of which serve as address lines AO–A5. These address lines serve as inputs to two BCD to 7-segment decoder/driver

(DM7447A, Fairchild Semiconductor, South Portland, ME) to control two LED displays indicating the number corresponding to a particular microelectrode site. A dual 2-to-4 line decoder provides enable lines EN₀–EN₂, which activate the appropriate analog multiplexer (8 channel: ADG408BN, 16 channel: ADG406BN, Analog Devices, Norwood, MA). Extracellular signals are then directed via the appropriate multiplexer to V_{OUT} . In addition to the multiplexer output, three additional channels can be selected for output via pin-to-pin assignment from the ribbon cable. The signals are low pass filtered with a 4-pole Bessel anti-aliasing filter (DP74L4L, Frequency Devices, Haverhill, MA) with a corner frequency of 5 kHz. Depending on the tissue preparation, extracellular potentials can range from 20 μ V to 1 mV [8]. Since typical 12-bit A/D converters with an input voltage range of ± 10 V have a sampling error of ± 4.88 mV, an overall system gain of at least 240 is required. To ensure that the extracellular signals can be distinguished, the four channel out-

puts are amplified with gains of 5 or 25 using LM6171 operational amplifiers (National Semiconductor) which are suitable for driving capacitive loads. The additional amplification yields a switchable overall system gain of either 1000 or 5000 at 1 kHz, more than sufficient for digitizing extracellular potentials without saturation.

2.4. Extracellular recording system performance

To evaluate the performance of the system, the power consumption, the frequency response of the amplifier channels, the input-referred noise, and the crosstalk between channels were assessed. The frequency response and channel crosstalk were obtained by using the swept sine function of a dynamic signal analyzer (model HP35665A, Hewlett Packard) yielding gain and phase data. The input-referred noise was

determined by grounding all of the system inputs, measuring channel output using an oscilloscope with digitizing and math functions (model 7854, Tektronix, Wilsonville, OR), and dividing by the amplifier channel peak gain. For quantification of system characteristics, the overall system gain was set to 1000 (60 dB). Where appropriate, data are given as mean $\pm$ standard deviation.

2.5. Cell culture

Embryonic day 11 chick cardiac myocytes (Truslow Farms, Chestertown, MD) were obtained by enzymatic dissociation using trypsin (Gibco-Life Technologies, Rockville, MD) and DNase I (Gibco-Life Technologies) of embryonic cardiac tissue as described earlier [9,10]. For a typical experiment, cells were plated at a

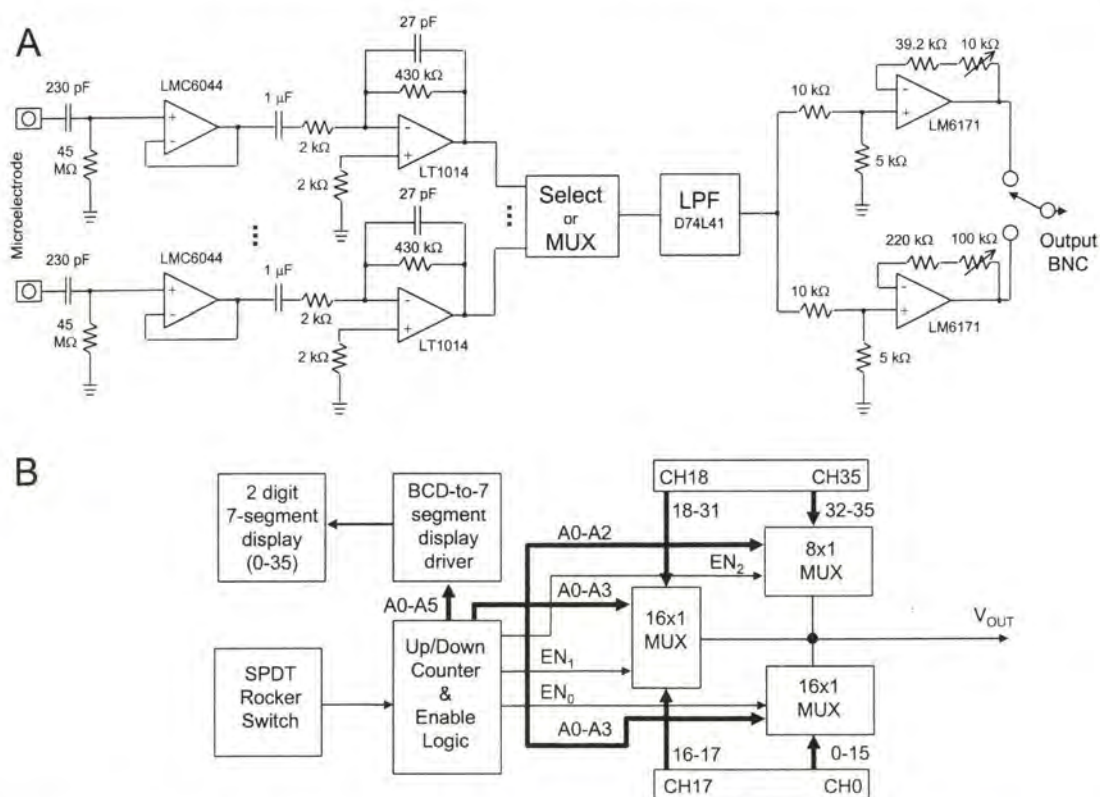


Fig. 3. Biosensor circuit and logic diagrams. A, extracellular potentials detected at the microelectrode sites are buffered via an ac-coupled follower with a corner frequency of 80 Hz, amplified by ~ 200 . The LMC6044 is a CMOS quad operational amplifier characterized by extremely low input leakage currents, making it ideal for portable, high source impedance amplifier applications. The buffered extracellular signal is amplified by the next stage using a LT1014, a quad precision operational amplifier exhibiting low offset voltages and high common-mode and power-supply rejection ratios. Amplifier outputs can be selected using a multiplexer (MUX) or directly from a ribbon cable connector. A selected channel is passed through an anti-aliasing, low-pass Bessel filter (LPF) with a corner frequency of 5 kHz. The filtered signal is amplified by 5 and 25 using LM6171 operational amplifiers which are well-suited for driving capacitive loads characteristic of ribbon cables. The filtered extracellular signal is amplified by a gain of either 1000 or 5000 and directed to the output BNC by a switch as shown. Resistors and capacitors were bin-sorted to be within 0.1 and 0.5% of design values, respectively. B, logic diagram for the multiplexer. A SPDT rocker switch drives two 4-bit up/down binary counters (DM54LS193, National Semiconductor), the binary output of which serve as address lines A0–A5. These address lines serve as inputs to two BCD to 7-segment decoder/driver (DM7447A, Fairchild Semiconductor) to control two LED displays indicating the number corresponding to a particular microelectrode site. A dual 2-to-4 line decoder provides enable lines EN₀–EN₂, which activate the appropriate analog multiplexer (8 channel: ADG408BN, 16 channel: ADG406BN, Analog Devices). Extracellular signals are then directed via the appropriate multiplexer to V_{OUT}.

density of 1×10^6 cells cm^{-2} . Culture media consisted of: 6% heat-inactivated bovine calf serum, 40% Medium 199 (M0650, Sigma), 0.1% Penicillin–Streptomycin (BRL 15140-122, Gibco), and 54% sterile-filtered low K^+ balanced salt solution (in mM): 116 NaCl, 0.8 NaH_2PO_4 , 1.18 KCl, 26.2 NaHCO_3 , 0.87 CaCl_2 , 5.5 dextrose. Myocardial cell viability based on trypan blue exclusion typically exceeded 90%. Cardiac cell monolayers were usually used within a week of dissection.

3. Experimental results

3.1. Extracellular recording system performance

The system can be operated either by battery or external power supply providing ± 16 V. Two battery packs provide power at the positive and negative rails of the main system comprising the buffer, amplifier, multiplexer, and anti-aliasing filter stages, whereas a separate battery pack is dedicated for thermal control. Under quiescent conditions, total power consumption reached 14 W yielding 2–3 h of system performance in an ambient temperature of 21–23°C.

Within the laboratory and the ac-coupled buffer inputs grounded, an output noise level of 8.8 ± 0.9 mV_{RMS} was measured, which corresponds to an input-referred noise of 8.7 ± 0.3 μV_{RMS} . Even with introduction of the microelectrode array bathed in 0.9% saline and the solution grounded through a gold wire, the noise level rose only slightly to 9.8 ± 0.3 μV_{RMS} . Furthermore, we assessed input referred noise in an outdoor environment which was only 10.6 ± 0.8 μV_{RMS} . Overall, the system noise levels compared well with the previously described amplifiers for extracellular recording; 15 μV [11], 10 μV [12,13].

The amplifier exhibited a bandpass frequency response over a frequency range suitable for extracellular recordings with low and high corner frequencies at 80 ± 2 Hz (mean $\pm$ STD) and 2.8 ± 0.1 kHz, respectively. At 1 kHz, the channel gain and phase shift were 59.9 ± 0.1 dB and $-36.4 \pm 0.8^\circ$, respectively. It should be noted that the low-pass Bessel filter anti-aliasing chip (Frequency Devices) can account for -24° phase shift at 1 kHz. A typical gain and phase shift profile is illustrated in Fig. 4A. To more accurately assess the impact of the microelectrode array on the system frequency response, a gold wire was introduced into the solution to drive the bath potential using the signal analyzer and the output was recorded to determine the frequency response. Addition of the microelectrode array resulted in very little change in the system response; low and high corner frequencies were 85 ± 3 Hz and 2.7 ± 0.1 kHz, respectively, and the gain at 1 kHz was 59.7 ± 0.2 dB.

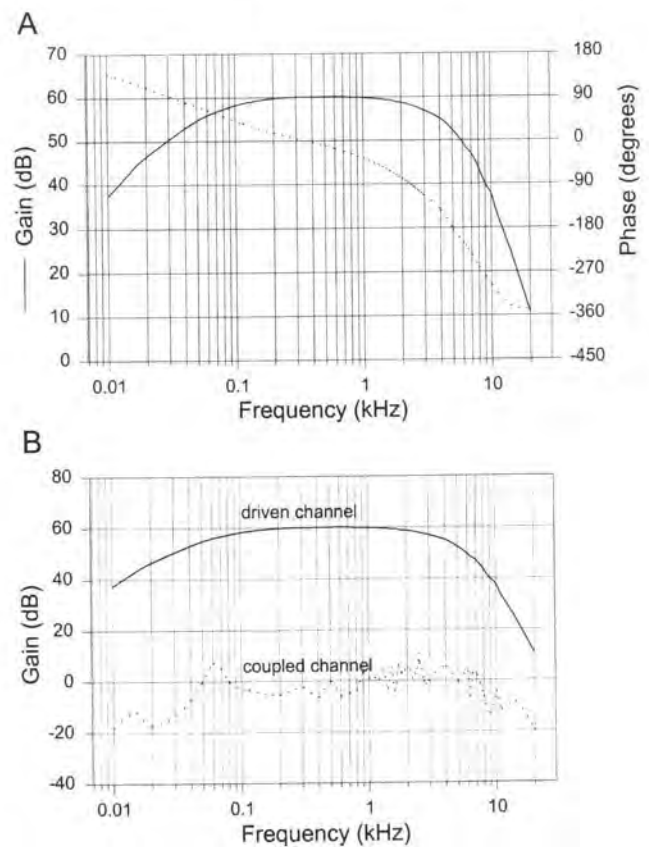


Fig. 4. Plots of the frequency response and crosstalk between channels. A, gain (solid) and phase (dotted) response of a typical amplifier channel. Over the frequency range of interest for extracellular recordings, 100 Hz to 2 kHz, the gain and phase vary from 57 to 60 dB and 45 to -80° , respectively. B, comparison of channel gain (solid) and crosstalk (dotted). Crosstalk between typical neighboring channels was assessed by measuring the output from channel A while driving the input to channel B and grounding the input of channel A.

For crosstalk measurements, we surveyed several different input channels. The input of one channel was driven with the signal analyzer while grounding the input of a second channel and recording the output of the second channel. As illustrated by the typical data in Fig. 4B, crosstalk gain was approximately 0 dB at 1 kHz reaching a maximum of approximately 10 dB at 3.0 to 3.7 kHz. For example, crosstalk for channel 3 was 2.03 ± 7.03 dB at 1 kHz reaching a maximum of 10.9 ± 3.1 dB over a range of frequencies, 3.4 ± 2.1 kHz. Relative to the driven channel, channel separation was -60 dB over the frequency range of interest. To evaluate the significance of the crosstalk measures, consider a maximum coupling of 11 dB and a biopotential of 1 mV at the input of a channel. One would expect a resulting 3.6 mV signal at the output of the coupled channel corresponding to a putative biopotential of only 3.6 μV . Therefore, any such crosstalk would fall below the input-referred noise of the amplifier.

3.2. Extracellular recordings of excitable cells

After 3–4 days in culture, chick cardiac myocytes exhibited a spontaneously pulsating monolayer. Consistent with earlier work [14–17], the extracellular cardiac potentials were multiphasic reminiscent of the second or third derivative of the action potential (Fig. 5A). Extracellular potentials were typically less than 1 ms in duration, occurring at a rate of 1–2 Hz with little variation in rate within a sample. For the experiment in Fig. 5A, propagation velocities between the microelectrode sites (shown in Fig. 1) ranged from 0.10 to 0.12 m s⁻¹. This range, which falls below that of a previous report [15], may be related to an irregular pattern of interconnectivity [16]. Nevertheless, delays between spikes at neighboring microelectrode sites were extremely consistent beat-to-beat. In five experiments, we observed complete cessation of extracellular activity with application of 300–500 μ M cadmium chloride or 30 μ M tetrodotoxin (TTX). Two further experiments conducted outdoors showed well-resolved extracellular potentials that were inhibited with the addition of TTX within 30 s (Fig. 5B). While these observations demon-

strate the physiological nature of the acquired signals, future work will address the subtle variations in extracellular waveforms, interspike intervals, and signal propagation in response to agents of interest.

4. Conclusion

To our knowledge, this is the first demonstration of system capable of performing a cellular function-based assay in the field. The major features of this prototype portable biosensor capable of extracellular recording from excitable cells have been described and characterized. System components that include an ac-coupled buffer, an amplifier stage, a multiplexer, anti-aliasing filters, and a thermal control system were constructed using low-cost, off-the-shelf components. The system has several important attributes that make it suitable for extracellular recording applications including an appropriate bandwidth for measuring extracellular potentials from a variety of excitable cells and a low crosstalk between channels. The prototype system provides only 2–3 hours of operation with the Ni–Cd battery packs; lithium batteries would provide a substantially longer operational window, albeit at a higher cost.

The microelectrode array, which was designed to be manufacturable and conducive for cell adhesion and function, permitted cardiac culture over a duration similar to that achieved under control conditions in polystyrene culture dishes. Planar microelectrode arrays manufactured using photolithographic techniques have been previously shown to be useful [8,18–20], but typically employ a 'zebra strip', a flexible silicone strip striated with conductive carbon, to achieve electrical contact between the pads of the planar array and the amplifier stage. The integration of the present array into a standard 40-pin DIP enables the array to be rapidly and easily replaced.

For system evaluation with excitable cells, planar microelectrode arrays were modified by adhering a 35 mm polystyrene culture dish with a hole drilled in the center so that cells would be easily maintained in a cell culture facility. Clearly, future work must address the need for a more sophisticated and rugged chamber interfaced with the planar microelectrode array. An enhanced chamber design would incorporate fluidic ports for controlled cell media perfusion and sample injection. Recent efforts in microfabrication have resulted in the development of an integrated silicon-polydimethylsiloxane cell cartridge that permits fluidic connections and may offer a suitable microenvironment for cell culture [21].

In summary, we have described and characterized a prototype portable cell-based biosensor. Our approach utilizes extracellular potentials from excitable cells

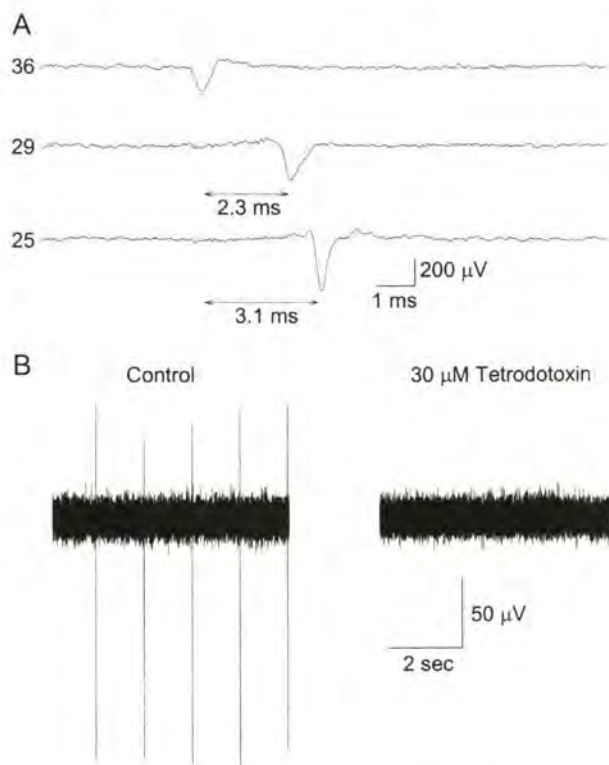


Fig. 5. Extracellular action potentials (APB) from a monolayer of cultured chick cardiac myocytes. A, simultaneous recordings from multiple microelectrode sites illustrating delay in AP propagation from microelectrode sites 25, 29, and 36. B, recording from a single representative microelectrode site before and after the administration of the sodium channel antagonist tetrodotoxin (TTX). TTX (30 μ M) induced a cessation of spontaneous firing after 30 s. Prior to application of TTX, the rate of spontaneous firing was 0.8 Hz.

recorded via a microelectrode array as a measure of cell functionality. The system incorporates temperature control, amplifier and filter stages to accommodate various excitable cell types, and a 36-channel multiplexer assembled from off-the-shelf components.

Acknowledgements

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SENSORS AND ACTUATORS

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APPENDIX E

CMOS microelectrode array for the monitoring of electrogenic cells

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Abstract

Signal degradation and an array size dictated by the number of available interconnects are the two main limitations inherent to standalone microelectrode arrays (MEAs). A new biochip consisting of an array of microelectrodes with fully-integrated analog and digital circuitry realized in an industrial CMOS process addresses these issues. The device is capable of on-chip signal filtering for improved signal-to-noise ratio (SNR), on-chip analog and digital conversion, and multiplexing, thereby facilitating simultaneous stimulation and recording of electrogenic cell activity. The designed electrode pitch of 250 μm significantly limits the space available for circuitry: a repeated unit of circuitry associated with each electrode comprises a stimulation buffer and a bandpass filter for readout. The bandpass filter has corner frequencies of 100 Hz and 50 kHz, and a gain of 1000. Stimulation voltages are generated from an 8-bit digital signal and converted to an analog signal at a frequency of 120 kHz. Functionality of the read-out circuitry is demonstrated by the measurement of cardiomyocyte activity. The microelectrode is realized in a shifted design for flexibility and biocompatibility. Several microelectrode materials (platinum, platinum black and titanium nitride) have been electrically characterized. An equivalent circuit model, where each parameter represents a macroscopic physical quantity contributing to the interface impedance, has been successfully fitted to experimental results.

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Keywords: Microelectrode array; CMOS; Electrogenic cells; Cardiomyocytes; Cell-based biosensor; Impedance

1. Introduction

Since its inception in the early 1970s (Wise, 1971; Thomas et al., 1972), the microelectrode array (MEA) has evolved into a tool capable of making significant contributions to the fields of neuroscience and biosensing (Rutten, 2002). The primary advantages of extracellular recordings over traditional methods (patch clamp, voltage-sensitive dyes) include non-invasiveness, multi-site measurement capabilities, and long measurement periods. However, despite concentrated research efforts widespread use has yet to be achieved as crucial design and development issues, such as low signal-to-noise ratio (SNR), flexible stimulation and readout capabilities, perfusion chambers with coupled microfluidics, and controlled growth techniques have yet to be satisfactorily addressed (Borkholder, 1998; Gross et al., 1995).

MEAs, with their ability to measure complex spatio-temporal neural activity, are poised to become a fundamental tool in neuroscience research.

In developing embryos, synchronized burst activity is thought to be important in fine tuning neuronal networks. MEAs have achieved a high degree of success in the study and characterization of this activity (Keefer, 2001a,b; Tschertter et al., 2001; Streit et al., 2001; Jimbo and Robinson, 2000). To study neurological phenomena such as Hebbian learning, the neural networks must be mapped; this could be achieved using neural tissue slices which exhibit significantly reduced cell mobility. Organotypic culturing of rat hippocampal and spinal cord tissue slices on MEAs (Egert et al., 1998; Thiebaud et al., 1999; Borkholder et al., 1997) demonstrate this potential. Other ways to create defined neural networks are to control the growth of processes with columnar structures (Zeck and Fromherz, 2001), with chemical cues (Prinz and Fromherz, 2000), or to trap the cells in cages (Maher et al., 1999). Shahaf and Marom have shown that learning in cortical neural networks occurs *in vitro*, thereby demonstrating the effectiveness of MEAs in the study of synaptic plasticity (Shahaf and Marom, 2001; Marom and Shahaf, 2002).

Cell-based biosensors are promising tools for the detection of chemical and biological toxins or for the screening of pharmacologically active compounds (the reader is directed

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to numerous reviews: Kovacs, 2003; McFadden, 2002; Pancrazio et al., 1999; Gross et al., 1995). These systems employ highly evolved cellular pathways and have the advantage that no previous knowledge of the investigated compound is required. Field-portable systems using cardiomyocytes have been developed at the Stanford Transducers Lab and the Naval Research Laboratory (DeBusschere and Kovacs, 2001; Gilchrist et al., 2001; Pancrazio et al., 1998b). Neuronal network-based biosensors have been used to demonstrate that *in vitro* cultures retain tissue-specific properties, and moreover, findings corroborate well with whole animal *in vivo* studies (Morefield et al., 2000; Keefer, 2001a,b). It can be envisioned that cell-based biosensors will replace animals in toxicology studies (van der Schalie et al., 1999).

The accurate measurement of low extracellular bioelectric signals, ranging from microvolts for neurons to millivolts for cardiomyocytes, at multiple points within a culture are the two key requirements considered when designing an MEA and recording system. Typical system designs incorporate an MEA with an external, off-chip system controlling electrode selection, signal amplification and interfacing (Gross, 1979; Gross et al., 1993; Borkholder, 1998; Connolly et al., 1990; Bucher et al., 1999; Heuschkel et al., 2002; Multi Channel Systems, 2001; Jenkner et al., 2001). However, as the recorded signal travels to the amplification system, passing several electronic interfaces, it can be distorted by electromagnetic interference resulting in a reduced signal-to-noise ratio. A crucial design consideration for large arrays is multiplexing. If multiplexing circuitry is not included each electrode requires an associated bond pad thereby limiting the array size to the number of available bond pads. CMOS integration can overcome both of these technical challenges. First approaches include a two-chip solution, where a CMOS chip with instrumentation amplifiers and electrode selection circuitry is placed in close proximity to the MEA chip (Pancrazio et al., 1998a). Single-chip solutions with fully-integrated CMOS multiplexing and buffering circuitry have been presented, with on-chip (Bai and Wise, 2001) and off-chip (DeBusschere and Kovacs, 2001) signal amplification. More recently systems have emerged, such as a FET-based, 16,384 microelectrode CMOS biosensor array comprising on-chip amplification circuitry with readout multiplexers and buffers (Eversmann et al., 2003), or, similarly, an MEA with on-chip amplification, filtering and addressability (Berdondini et al., 2002). While these designs represent significant improvements in neurochip development and the use of multiplexers translates into almost unconstrained array sizes, they do not yet include flexible on-chip stimulation capabilities.

Here, we report on a new biochip comprising a microelectrode array and fully-integrated analog and digital CMOS circuitry for the stimulation and recording of activity from electrogenic cells. In contrast to the systems mentioned previously, this device is capable of on-chip signal filtering for improved SNR, on-chip analog and digital conversion to prevent signal degradation, multiplexing, and

simultaneous recording and stimulation. Each electrode has an associated circuitry unit including a stimulation buffer and switch, a bandpass filter and a readout buffer. The electrode and circuitry form a repeatable unit with a pre-defined 250 μm pitch that significantly limits the area available for circuitry. To achieve the electrode pitch, only the components necessary to condition the small signals and to provide reliable, addressable stimulation signals have been assembled in a repeated circuitry unit. This repeatable unit can be multiplied to form a larger array. The stimulation buffer uses a class-AB output stage in order to deliver large currents to the electrode. Stimulation voltages are generated from an 8-bit digital signal converted to an analog signal at a frequency of 120 kHz. As a result, any stimulation waveform can be generated. The bandpass filter has corner frequencies of 100 Hz and 50 kHz and a gain of 1000. The microelectrode is realized in a shifted design for flexibility and biocompatibility purposes. Several biomedically relevant microelectrode materials, platinum, platinum black and titanium nitride, have been electrically characterized, and an equivalent circuit model of the impedance is presented.

2. Methods

2.1. System design

The 4.4 mm $\times$ 4.4 mm chip presented here consists of a 4 $\times$ 4 electrode array with integrated reference electrode (Fig. 1) (Franks et al., 2003). Fabrication was performed using an industrial double-polysilicon, triple-metal, 0.6 μm CMOS process at Austriamicrosystems AG (Austria). The electrodes are 40 μm $\times$ 40 μm with a 250 μm pitch. Space between the electrode array and bond pads has been intentionally left blank for the sealant that isolates the cells from the bond wires. A block diagram with the main circuitry components is given in Fig. 1. A unit of repeating circuitry comprising a buffer and switch for stimulation, a bandpass filter and buffer for readout, is provided with each electrode (inset, Fig. 1). Additionally, the system architecture incorporates four analog-to-digital converters (ADC, one per row of electrodes) and one digital-to-analog converter (DAC). On-chip multiplexing and A/D-conversion allow for a reduction in the number of bondpads, which facilitates the integration of a large array. A digital control unit scans the array during readout, provides individual electrode addressability for stimulation, and interfaces with the outside world. Simultaneous recording and stimulation at each electrode can be performed. For the purpose of monitoring the chip operation temperature and the culture temperature, a temperature sensor has been included.

2.2. Electrode design

Due to a limited selection of materials available in a commercial CMOS process, the electrode material as received

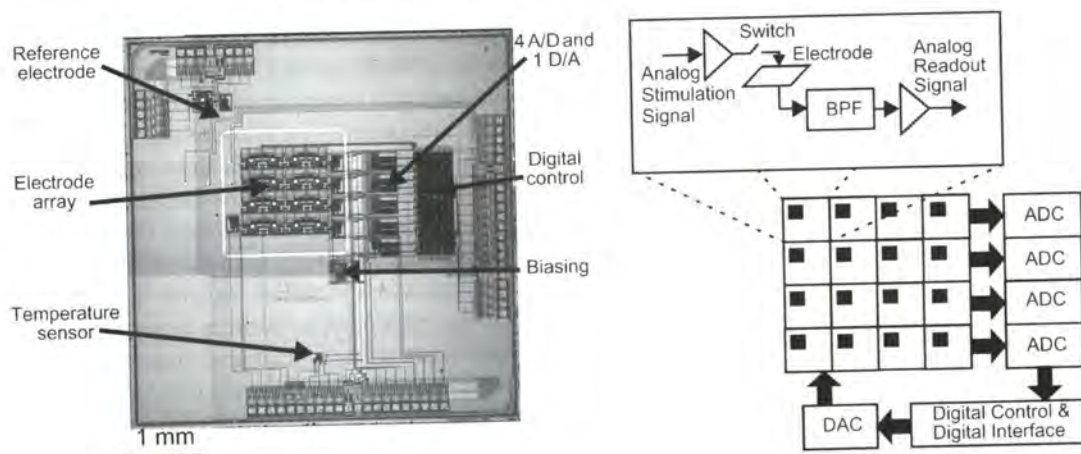


Fig. 1. Photograph of CMOS chip on the left. Block diagram of the system architecture on the right. Inset shows the contents of the repeated circuitry unit.

from the foundry is aluminum, a known neurotoxicant. A simple 2-mask post-processing procedure is employed to cover the Al electrodes with biocompatible platinum. During this processing the electrodes are shifted (Fig. 2), and the aluminum is sealed with the electrode metal and a passivation stack. It is necessary to seal the underlying Al to avoid both poisoning and undesirable electrochemistry that occurs when the Al comes into contact with the neuron medium. The electrode metal consists of 50 nm TiW, an adhesion promoter, and 270 nm of Pt. The metals are sputter-deposited and structured in a lift-off process. An alternating $\text{Si}_3\text{N}_4/\text{SiO}_2$ passivation stack consisting of 1 μm of Si_3N_4 , and two times 100 nm SiO_2 and 200 nm Si_3N_4 (total layer thickness of 1.6 μm) is deposited using plasma-enhanced chemical vapor deposition (PECVD). A mixed frequency PECVD process was used to match the

stress with that of the underlying Si_3N_4 deposited during the CMOS process. A simpler solution featuring a single 1 μm Si_3N_4 layer has been attempted, but proved to be ineffective in sealing the Al, resulting in the dissolution of the reference electrode during cell culturing. It is believed that the roughness of the underlying Al layer leads to pinholes in the Si_3N_4 layer; however, when a stack of alternating oxide and nitride layers is deposited these pinholes are covered. Neurons have been cultured for 28 days on chips with the 1.6 μm passivation stack, and no degradation effects have been observed. A reactive-ion etch (RIE) is then used to open the nitride stack defining the size and shape of the electrodes. This increases design flexibility since the electrode dimensions are not defined by the CMOS process. The processed chips are bonded and packaged in a two-step process. An epoxy (Masterbond 42HT-T, Masterbond Inc., USA) is first used to stabilize the bonds on the chip and the package. Polydimethylsiloxane (PDMS) is then used to form a bath, isolating the bond wires from the cells. A glass O-ring, affixed to the chip with PDMS, forms a larger bath capable of containing a suitable amount of neuron medium (Fig. 3).

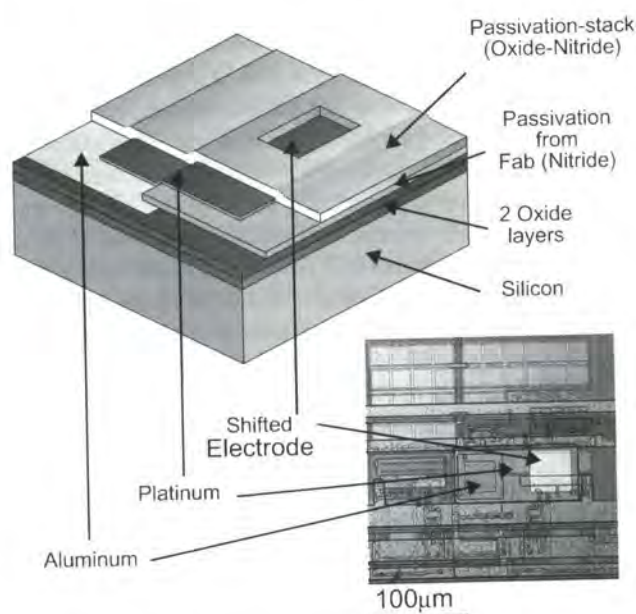


Fig. 2. Cross-section (left) and micrograph (right) of the shifted Pt-electrode. Micrograph shows one circuitry repeating unit.

2.3. Electrode characterization and modeling

Extracellular signals are typically minute making a low electrode–electrolyte interface impedance imperative. To characterize the electrode–electrolyte interface electrochemical impedance spectroscopy (EIS) has been performed for bright Pt, Pt black and TiN. The EIS measurements have been used to model the interface with an equivalent circuit model where each parameter represents a macroscopic physical process contributing to the interface impedance. An enhanced understanding of the physical processes can be used to better understand the transduction principle and to design a lower-impedance electrode.

The measurements have been fitted to the equivalent circuit model (Fig. 4) comprising a constant phase angle impedance, Z_{CPA} , shunted by a charge transfer resistance,



Fig. 3. Image of packaged chip.

R_{CT} , together in series with the solution resistance, R_s (Grahame, 1952). The constant phase angle impedance first proposed by Frick (1932) is a measure of the non-Faradaic impedance arising from the interface capacitance, or polarization capacitance, and is given by the empirical relation:

$$Z_{CPA}(\omega) = \frac{1}{(j\omega Q)^n}$$

where Q is a measure of the magnitude of Z_{CPA} , n is a constant ($0 \leq n \leq 1$) representing inhomogeneities in the surface and $\omega = 2\pi f$. In a Nyquist plot, the angle between the data and the abscissa axis gives n from $n = (2\theta)/\pi$. When $n = 1$, Z_{CPA} represents a pure capacitive element corresponding to the interface capacitance. Faradaic charge transfer leads to R_{CT} , which in the case of Pt is believed to be due to the hydrolysis of H_2O and reduction of O_2 according to $2H_2O \leftrightarrow O_2 + 4H^+ + 4e^-$ where the equilibrium potential is $0.987 - 0.059 \times \text{pH}$ with respect to the standard calomel electrode (SCE) (Yeager, 1984). Experiment findings support this assumption. When the open-circuit potential (OCP) is set to value higher or lower than the equilibrium value of ~ 0.6 V, then the R_{CT} is lower than if the OCP were equal to the equilibrium value. The higher or lower potential shifts the reaction from equilibrium conditions, more Faradaic charge transfer occurs, and R_{CT} is reduced. For example, for an OCP of 0.66 V the charge transfer resistance is 1.0 M Ω . For an

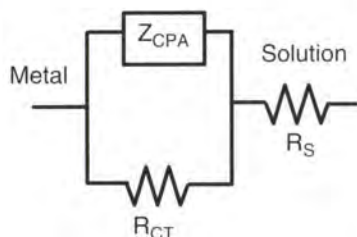


Fig. 4. Equivalent circuit model of electrode-electrolyte interface. Measured results are given in the table.

	Q	n	R_s	R_{CT}
	[s $\Omega^{-1/n}$]		[Ω]	[10 ⁵ Ω]
Pt	2.72(10) ⁻⁵	0.92	28.0	4.48
Std. Dev.	0.26(10) ⁻⁵	0.01	2.81	0.88
Pt Black	2.08(10) ⁻³	0.91	30.7	0.51
Std. Dev.	0.11(10) ⁻³	0.03	7.85	0.28
TiN	2.03(10) ⁻³	0.91	42.3	3.00
Std. Dev.	5.77(10) ⁻⁵	0.00	3.70	3.20

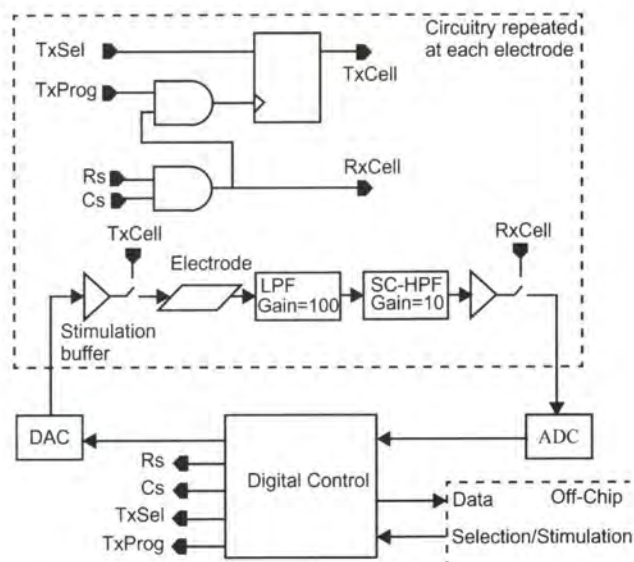


Fig. 5. Schematic of stimulation and readout circuitry.

OCP of 0.39 and 0.92 V the charge transfer resistance reduces to 0.43 and 0.23 M Ω , respectively. However, it should be noted that the presence of any impurities at the interface will also lead to a reduced charge transfer resistance.

2.4. Stimulation circuitry

The chip presented in this paper is capable of stimulating any subset of electrodes with any arbitrary waveform. When a stimulation phase is enabled, the on-chip digital control sets the selection by adjusting the switch between the stimulation-buffer and the electrode (Fig. 5). Since the impedance of the electrode varies significantly with electrode material, size, and electrolyte, a buffer with a class-AB output stage was used in order to be capable of delivering large currents (up to 10 mA if needed). The buffer (Fig. 6) comprises a differential input stage (M1, M2) with an active load (M3) and is connected in unity gain (V_{OUT} connected to gate of M2). The class-AB stage is formed by the transistors M5, M6, M9 and M10, where M5 and M6, both diode connected, bias the output transistors M9 and M10. If the input voltage (V_{IN}) is increased, M9 forces the output voltage (V_{OUT}) to follow. The load current is delivered by

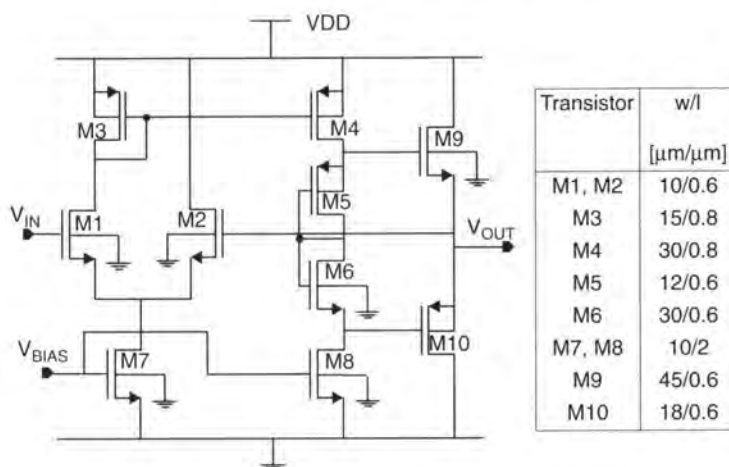


Fig. 6. Schematic of the class-AB stimulation buffer. Transistor sizes are summarized in the table.

M9, whose gate-source voltage (V_{GS}) will increase slightly. Since a constant voltage is maintained between the gates of M9 and M10 the V_{GS} of M10 decreases by the same amount that the V_{GS} in M9 increases. M10 stays on, but conducts only a small current. When V_{IN} is lowered the situation is inverted, with M10 forcing V_{OUT} to follow and with the current through M9 decreasing. The power consumption is $45 \mu\text{W}$ for vanishing input, given a current of $2 \mu\text{A}$ through M7 and M8, and a supply voltage (V_{DD}) of 5 V . However, when slewing, the amplifier can deliver up to 10 mA to the electrode. This corresponds to a maximum current density of 60 A/cm^2 . The whole buffer occupies an area of $42 \mu\text{m} \times 32 \mu\text{m}$ and has a bandwidth of $\sim 10 \text{ MHz}$.

The programming of the array is described as follows (Fig. 5). Transmission gates are used as switches for stimulation (TxCell) and readout (RxCell). Both are set by a small control unit consisting of a flip-flop and two AND gates. The row and column signals, RS and CS, are used for readout operation. When RxCell is high the signal is sampled by the ADC.

The programming of the array is done with two signals TxSel and TxProg. The on-chip digital control generates these signals based on the pattern information received from the PC. When RxCell goes high while TxProg is high the flip flop, which controls the stimulation switch, can be set with TxSel.

Subsequent to selection digital stimulation patterns are sent to the chip continuously until the stimulation phase is stopped. Stimulation voltages are in a range given by two reference voltages, which have a resolution of 8-bit and a cycling of 120 kHz . The time delay between enabling a stimulation phase and first stimulation voltage to reach the electrode is $25 \mu\text{s}$. The time required to go from one subset of electrodes to the next including deselection, selection and time required for a first stimulation voltage to reach the electrodes is $50 \mu\text{s}$. Stimulation phases can take place at any time throughout the measurement and are not limited in number. For deselection of the electrodes the signal TxProg is set high while TxSel is low.

2.5. Readout circuitry

Electrogenic cells induce small signals on the electrodes. The range is tens of microvolts for neural signals up to millivolts for heart cells. In order to prevent signal degradation signal conditioning should be implemented as close to the electrode as possible. For this reason, buffering and filtering circuits are adjacent to and repeated at each electrode (Figs. 1 and 5). The available space for the circuitry blocks is limited by the pitch between two electrodes, which is designed to be $250 \mu\text{m}$. It is possible that several cells produce signals on the same electrode. A 20 kHz sampling frequency is adopted so as to be able to resolve signal components originating from different cells.

When an action potential occurs in a cell above the electrode, ions flowing across the cell membrane inducing a charge redistribution on the electrode. The frequency of these signals is expected to be approximately 1 kHz . AC-coupling is mandatory for biological signals due to large drifts. Additionally, low pass filtering limits the noise bandwidth and provides anti-aliasing. Consequently, we implemented a bandpass filter with a center frequency around 1 kHz . The corner frequencies of the filter were chosen to be 100 Hz and 50 kHz . Similar specification can be found in literature (DeBusschere and Kovacs, 2001; Bai and Wise, 2001). The bandpass filter is divided into two stages. The first step is a low-pass filter, which buffers and amplifies the signals by a factor of 100. The electrode is directly connected to the non-inverting input of the amplifier, the gate of a MOSFET, which has a high input impedance in CMOS technology. This is similar to other systems, which have successfully acquired signals from electrogenic cells (Fromherz, 2002, DeBusschere and Kovacs, 2001). Signals in the range of tens of microvolts can be measured since the equivalent input noise of the amplifier is below $10 \mu\text{V}_{\text{RMS}}$ over the band 100 Hz – 100 kHz . Very little area ($120 \mu\text{m} \times 70 \mu\text{m}$) is occupied by the amplifier. Furthermore, the amplifier uses an internally compensated OpAmp, which acts as a low-pass filter with a corner frequency of

~50 kHz. This corner can vary between 20 and 100 kHz due to process parameter variations. The low-pass transfer function in this first stage provides the anti-aliasing, that is mandatory for the switched-capacitor circuit in the following stage. The high-pass filter implemented in the second stage has a gain of 10 and a corner frequency at 100 Hz. A large time constant is needed in the high-pass filter, in order to achieve the low cut-off frequency of 100 Hz. Since the available space is limited, we used a switched capacitor solution. After the second filter, the signal is buffered and sent to the ADC via an analog multiplexer. As multiplexing takes place after signal amplification and filtering, the design is less sensitive to switching and electromagnetic interference. The ADC uses the successive-approximation technique and has a resolution of 8 bits. A positive and a negative reference voltage define the range. Digital values are finally sent to a PC through an 8-bit parallel bus.

3. Results

3.1. Electrode impedance

Fig. 7 shows the measured (dashed markers) and modeled (solid line) results of the impedance modulus and phase as a function of frequency for three different materials: bright Pt ($n = 4$), Pt black ($n = 4$), and TiN ($n = 3$). A large geometric surface area, 1 cm^2 , was initially used to avoid complications arising from low current measurements typical of very small areas. Measurements were performed using a commercially available Autolab PGSTAT30 potentiostat system with Frequency Response Analysis software (version 4.8, Eco

Chemie B.V., Netherlands). In this three electrode system a standard calomel electrode (SCE) is the reference electrode, the counter electrode is a large-area Pt-mesh, and the electrolyte is 0.9% NaCl. The perturbation potential was 10 mV and the scan range was 10^{-3} to 10^5 Hz. The Pt electrodes were additionally treated by voltammetric cycling from -0.5 to 1.0 V for typically six cycles, when the measurement stabilized. This procedure potentially results in the formation and reduction of Pt oxide and Pt dioxide layers, as indicated by the Pt–H₂O Pourbaix diagram (Pourbaix, 1974). Before performing the impedance scan the open-circuit potential (OCP) was recorded. The OCP is a function of the chemical composition of the interface, and can be used for quality control of the initial interface conditions. Average OCP values for Pt, Pt black and TiN were 0.318 ± 0.037 , 0.299 ± 0.061 , and 0.046 ± 0.034 V, respectively.

3.2. Equivalent circuit model parameter results

The derived values for the equivalent circuit model parameters, and standard deviation, are given in the table in Fig. 4. The various model parameters can be graphically interpreted from specific regions in the EIS measurements. At high frequencies the phase angle tends to zero and the modulus is solely due to R_s . Similarly, at low frequencies the phase once again tends to zero, and the modulus represents the sum of R_s and R_{ct} . In the mid-frequency range the phase tends to -90° , representing a capacitive element, in this case the interface capacitance. The deviation of the model from experimental results at low frequencies can be attributed to the non-linear behavior of R_{ct} at low frequency (McAdams and Jossinet, 1994). As expected, the dendritically structured Pt black and TiN, with an increased total surface area, reduce the impedance modulus by approximately two orders of magnitude. The impedance of $900 \mu\text{m}^2$ Pt black micro-electrodes was found to be $4.84 \times 10^4 \Omega$ at 1 kHz.

Cyclic voltammetry was used to determine the equilibrium exchange current density of bright Pt. The following measurement parameters were used: 5 mV perturbation signal with respect to OCP, 0.5 mV/s scan rate, 0.15 mV step potential, averaged over 10 scans. From the slope of the current-versus-applied-potential plot the exchange current density is calculated to be $8.5(10)^8 \text{ A/cm}^2$ and R_{ct} is $3.0(10)^5 \text{ } \Omega$. This finding results in the omission of a Warburg impedance element in the model over the measurement frequency range used here.

The presented model is similar to others that have been previously applied, however here the parameters are associated with macroscopic physical processes contributing to the interface impedance (De Boer and Van Oosterom, 1978; Onaral and Schwan, 1982). Models that do attribute physical processes to circuit elements include a Warburg impedance element for diffusion (McAdams et al., 1995; Kovacs, 1994). In the frequency range used here no experimental evidence for a diffusional impedance has been found and it has therefore been omitted. It is possible that a diffusional impedance

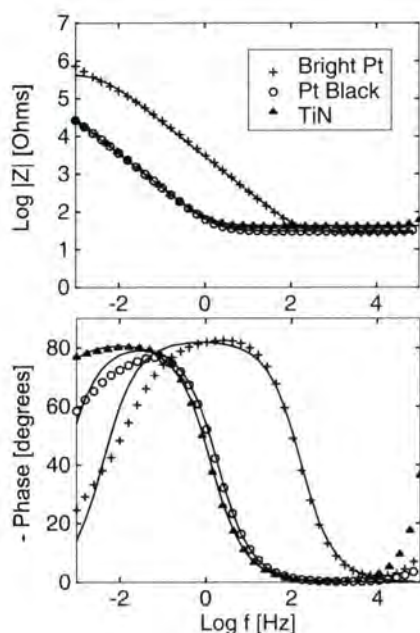


Fig. 7. Impedance modulus and phase as a function of frequency for 1 cm^2 Pt, Pt-black and TiN electrode materials.

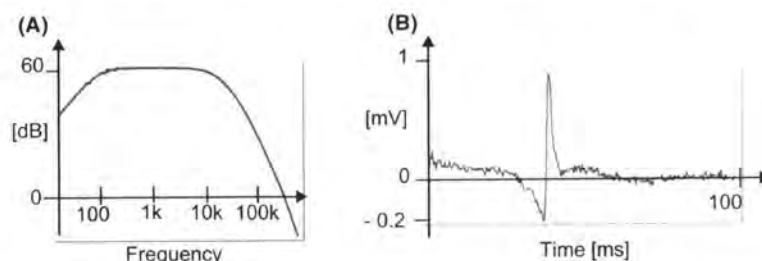


Fig. 8. Transfer function of the bandpass filter (A). Typical spike from embryonic chicken cardiomyocytes. High frequencies were cut-off at 3 kHz (B).

would reveal itself at lower frequencies, which will be the subject of future investigations. Additionally, it is interesting to note that n , often termed the frequency dispersion coefficient, does not vary with surface roughness, as has been historically assumed (De Levie, 1965). The effect of the surface roughness on impedance has been the focus of intense research efforts and has resulted in many models such as the well known pore model of de Levie, models based on surface inhomogeneities leading to a distribution of relaxation times (Brug et al., 1984), and more recently in fractal geometry-based models (Nyikos and Pajkossy, 1985; Bates and Chu, 1992). The authors concur with the work of Pajkossy, where in the frequency range investigated the capacitance dispersion is attributed to adsorption effects (Pajkossy, 1994).

3.3. Circuitry characterization

Stimulation circuitry operation is demonstrated by generating a square-wave stimulus on-chip. The resulting signal on a selected electrode was measured using a probe tip. The bandpass filter has been electrically characterized, using a gain-phase analyzer (HP-4194A), with results shown in Fig. 8A. Corner frequencies were measured at 95 ± 5 Hz and 72 ± 10 kHz. The large error in the upper corner frequency was expected because it is due to the dominant pole of the operational amplifier of the first stage. A gain of 59.2 ± 0.3 dB was measured at 1 kHz. The equivalent input noise of the filter is below 9 mV_{RMS} (100 Hz–100 kHz), as was measured with a spectrum analyzer (HP-4195A).

The performance of the temperature sensor was also measured. The temperature was swept from 20 to 50 °C and a 2-point calibration was performed. The measured sensitivity is about 210 mV/°C and the error is less than ± 0.2 °C.

3.4. First biological measurements

First tests were performed with an in situ preparation of cardiomyocytes from fertilized chicken eggs at embryonic day 10. The hearts were extracted from the embryo, briefly rinsed with cold Dulbecco's PBS (Sigma D-8531, Ca²⁺ and Mg²⁺-free) and directly transferred onto the electrodes of the chip. A typical spike is shown in Fig. 8B. DC-offsets at the electrodes were measured to be up to 1 V, which could cause saturation in the first amplification

stage. Since the time-constant of the electrochemistry at the electrode–electrolyte interface is on the order of seconds, we used the on-chip stimulation circuitry to set the electrode to a working point.

4. Conclusion

An extracellular monolithic recording system, with 16 platinum electrodes, fabricated in industrial CMOS-technology combined with post-CMOS processing has been presented. The circuitry architecture is structured in a modular design where a repeatable circuitry unit comprises the signal transducing electrode, a bandpass filter for immediate signal conditioning, and a buffer for stimulation. The system incorporates data converters and a digital control unit that scans the array and provides a digital interface to the outside world. Functionality of the circuitry components according to initial specifications has been verified. We showed that the chip is able to measure signals from electrogenic cells using embryonic chicken cardiomyocytes. Electrochemical impedance spectroscopy has been used to experimentally verify an equivalent circuit model of the electrode–electrolyte interface. The representation of the macroscopic physical processes occurring during the impedance measurement by the model parameters aids in the understanding of signal transduction, and may lead to an optimized transducer design.

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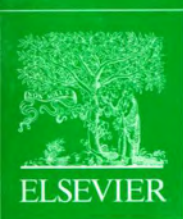
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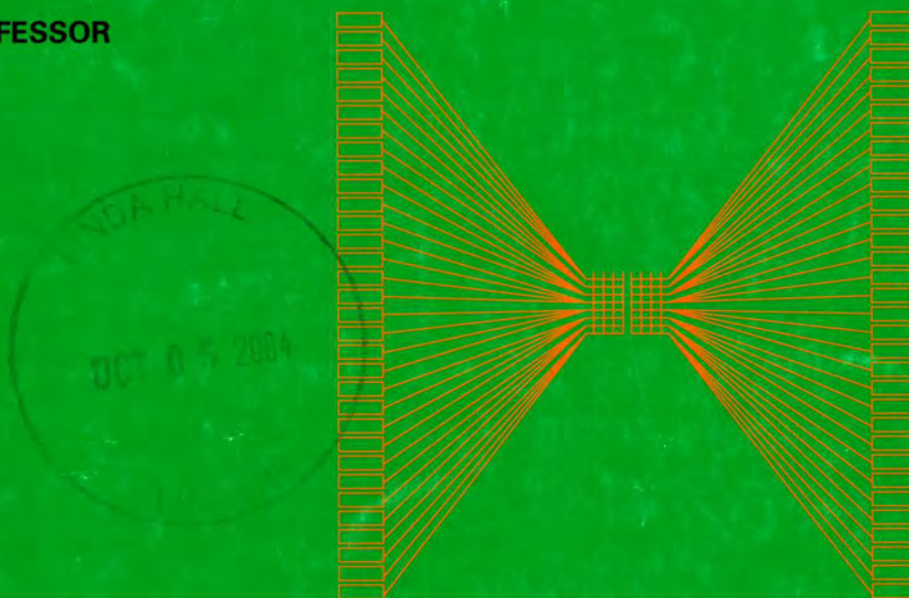
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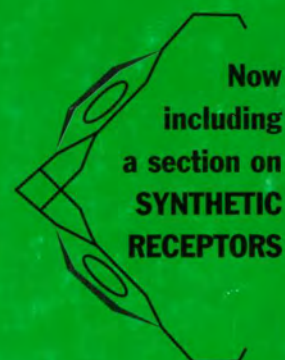
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APPENDIX F

A novel microfluidic impedance assay for monitoring endothelin-induced cardiomyocyte hypertrophy

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Abstract

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death. At the level of individual cardiac myocytes (heart muscle cells), the cell morphology alters (increase in cell size and myofibrillar re-organization) and protein synthesis is activated. In this paper, a novel cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis cell concentration is reported to monitor the dynamic process of endothelin-1-induced cardiomyocyte hypertrophy. A dielectrophoresis (DEP) microfluidic device is fabricated capable of concentrating cells from a dilute sample to form a confluent cell monolayer on the surface of microelectrodes. This device can increase the sensitivity of the impedance system and also has the potential to reduce the time for detection by a significant factor. To examine the feasibility of this impedance sensing system, cardiomyocytes are treated with endothelin-1 (ET-1), a known hypertrophic agent. ET-1 induces a continuous rise in cardiomyocyte impedance, which we interpret as strengthening of cellular attachments to the surface substrate. An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

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Keywords: Impedance analysis; Cardiac hypertrophy; Endothelin-1

1. Introduction

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death (Levy et al., 1990; Ruskoaho, 1992). Cardiac hypertrophy is defined as an increase in heart size and/or myofibrillar volume without a change in myocyte number. At the level of individual cardiac myocytes, the cell morphology alters (increase in cell size and myofibrillar re-organization) and protein synthesis is activated (Lorell and Carabello, 2000; Diane et al., 2000). Currently, the cardiac myocyte hypertrophy, *in vitro*, is monitored using a combination of radiological labeling, immunofluorescence labeling and kinase activity assessment based on morphological and immunochemical characteristics. The whole

process is invasive, time-consuming and unable to monitor the whole dynamic process. Therefore, it is necessary to develop a novel bio-analytical sensor device to obviate these cumbersome experimental assays and monitor cardiac myocyte hypertrophy non-invasively and in real time.

It has long been recognized that the membrane of biological materials, such as cells, exhibits dielectric properties. This biological property is exploited by the emerging sensor technology referred to as electric cell-substrate impedance sensing (ECIS), to monitor the morphology, viability, and environmental change for the adherent cells (Giaever and Keese, 1991, 1993). The use of living cells as sensor elements provides the opportunity for high sensitivity in a broad range of biologically active substances that affect the response of the cells. In ECIS, a current is applied through cultured cells plated on gold electrodes. Upon the attachment and spreading of cells on the gold electrodes, the impedance increases because the cells act as insulating particles to restrict the flow of current. The change

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ing impedance can be continuously monitored and interpreted to reveal information about cell spreading and micromotion (Ehret et al., 1997; Kowolenko et al., 1990). Impedance measurements have been used to monitor the behavior of many cell types such as macrophages (Mitra et al., 1991), endothelial cells (Xiao et al., 2002; Tiruppathi et al., 1992) and fibroblasts (Luong et al., 2001). Arndt et al. (2004) used endothelial cells as the sensor elements to study the cycloheximide-induced *apoptosis*. The feasibility of glucose sensing by fibroblasts over the physiological range of glucose concentrations was also demonstrated and shown to be inhibited by addition of cytochalasin B (Tlili et al., 2003).

In this work, a new cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis cell concentration has been developed. When cultured *in vitro*, cardiac myocytes are up to $20\ \mu\text{m} \times 100\ \mu\text{m}$ in size and can form close contacts to the substrate. Both of these characteristics are favorable for the development of high seal resistances. Thus, they make an excellent choice for impedance studies where a large cellular size compared to the extracellular electrode diameter is desirable. To further refine our impedance sensing system, a dielectrophoresis (DEP) microfluidic device has been fabricated capable of concentrating cells from a dilute sample to form a confluent cell monolayer. This device can increase the sensitivity of the impedance system and also has the potential to reduce the time for detection by a significant factor. To test the feasibility and practicality of our impedance sensing system, we treat cardiomyocytes with endothelin-1 (ET-1), which is known to induce cardiac myocyte growth (hypertrophy). An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

2. Materials and methods

2.1. Cell isolation and culture

Left ventricular (LV) myocytes were isolated and permeabilized according to a previously described protocol (Lim et al., 2001). Male Wistar rats, weighing between 200 and 250 g, were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and heparinized (200 IU i.v.). The hearts were rapidly excised and arrested in ice-cold cardioplegic Krebs Bulbring (KB) solution (85 mmol/L KOH, 30 mmol/L KCl, 30 mmol/L KH_2PO_4 , 3 mmol/L MgSO_4 , 0.5 mmol/L EGTA, 10 mmol/L HEPES, 50 mmol/L L-glutamic acid, 20 mmol/L taurine, 10 mmol/L 2,3-

butanedione monoxime, and 10 mmol/L glucose). The hearts were cannulated and retrogradely perfused via the aortic root with 1.2 mmol/L Ca^{2+} Tyrode solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2 , 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4), followed by Ca^{2+} free Tyrode solution until the hearts ceased beating. Hearts were then perfused with a digestion solution containing 0.08% collagenase A (Boehringer) and 0.02% protease XIV (Sigma–Aldrich). All perfusion solutions were equilibrated with 100% oxygen and maintained at 37°C throughout the isolation process. The digestion solution was eventually washed out with KB solution, and the hearts removed from the cannula. The LV was separated, minced, and gently agitated allowing the myocytes to be dispersed in KB solution. The myocyte suspension was subsequently filtered through a nylon mesh, resulting in a typical yield of >90% rod-shaped cells. The isolated cells were then washed with DMEM supplemented with albumin, creatine, carnitine, and taurine (ACCT media), with the addition of ascorbic acid (100 μM) and were either directly plated onto the microchamber with the microelectrodes precoated with laminin (Becton–Dickinson) or injected into the chamber for DEP capture of myocytes (see below).

2.2. Design and fabrication

In this task, glass microscope slides (2 in. $\times$ 3 in., Fisher Scientific) were covered with a layer of positive photoresist (AZ 5214) at 4000 rpm by means of a spin-coater; the resist was then hardened at 100°C for 6 min on a hot plate. After baking, the photoresist was patterned with a mask aligner using an exposure dose of 10 mW for 20 s, and subsequently developed by AZ 400 MIF developer (1:3) for 35 s. After this step, the glass slides were cleaned by DI water and dried by nitrogen. The first metal layer, for the DEP electrodes was deposited by sputtering of aluminum and patterned by liftoff to create the DEP electrodes. Then a thick layer of silicon dioxide was deposited by plasma enhanced chemical vapor deposition (PECVD) to completely isolate the DEP electrodes. Windows were defined in the PECVD oxide layer using conventional lithography and opened by reactive-ion-etching to access the bond pads on the first metal layer. Subsequently, a gold layer over a chromium adhesion layer was deposited by sputtering and patterned by lift off methods to create the measurement electrodes. The glass slide with microelectrodes was sealed with a custom-made silicone chamber with inlet and outlet tubes. An HP 33120A arbitrary waveform gener-

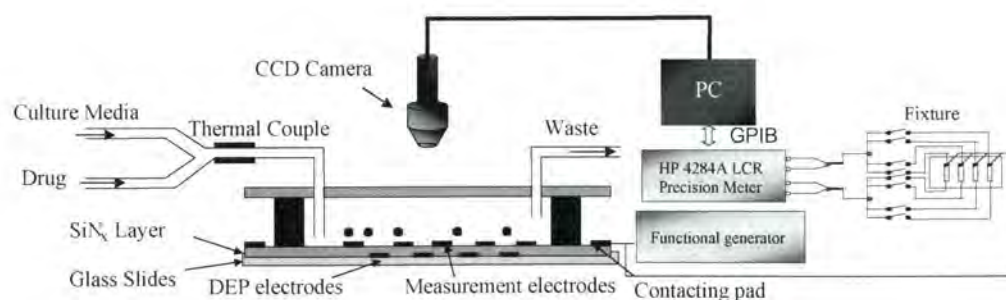


Fig. 1. Simplified crosssection of experimental set-up.

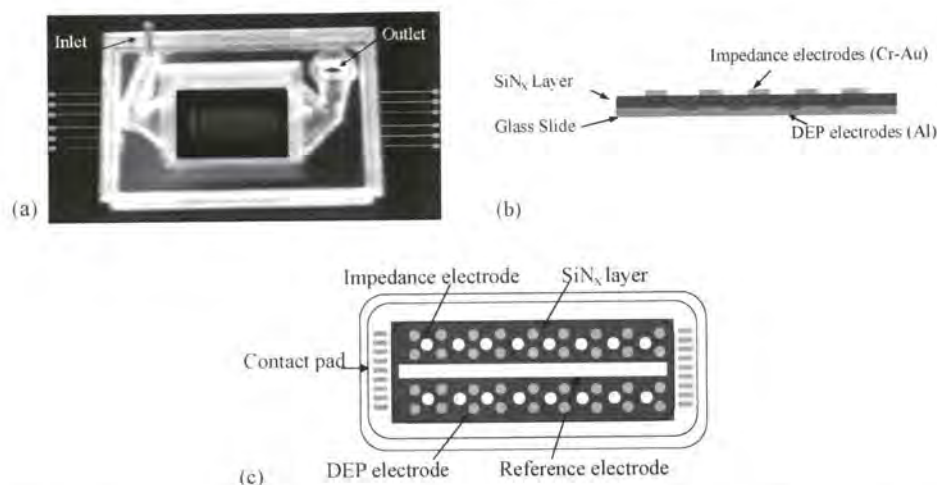


Fig. 2. (a) Photograph of device with flow chamber; (b) scheme of chip layer; (c) scheme of chip top view.

ator was used as the ac signal source to produce sinusoidal signal with a frequency specified at 2 MHz. Images were collected using a Nikon microscope and recorded by a charge-coupled device (CCD) digital camera.

2.3. Experimental set-up

The scheme of the experimental set-up for dielectrophoresis concentration and impedance measurements is presented in Fig. 1. A polycarbonate flow perfusion chamber with an internal volume of 30 μL sealed onto the biochip was used for all measurements (Fig. 2a). Schematic cross-section of chip layers and top view of the biochip are presented in Fig. 2b and c. DEP electrodes were used to concentrate cells from a dilute sample to form the cell monolayer and impedance electrodes (Cr–Au) were used to monitor the cardiomyocyte hypertrophy after the cell monolayer formation. Myocytes were injected into the chamber at a constant rate from one end using a motor driven micropump, and superfused with modified DMEM buffer solution. The flow rate in the microfluidic channel of the biochip was controlled by adjusting the pressure drop between the inlet and the outlet through a microflow controller (Cell Micro Controls Ltd., VA).

Experiments with the chip assembly were performed inside a custom-made heated platform with a built-in RTD temperature sensor. The chip assembly was completely enclosed to prevent the air flow disturbance. A temperature control system (Cell Micro Controls Ltd., VA) linked to the temperature sensor was used to keep a constant temperature of 37 $^{\circ}\text{C}$. The carbon dioxide concentration inside the chamber was maintained at 5% and humidified to prevent excessive evaporation of the medium. Electrical connections to the chips were established by using probes mounted on micromanipulators attached to the heated platform. This chamber with all of its components was capable of ensuring cell viability over long periods of time and stabilizing cell physiology in the absence of chemical agents.

The impedance of the electrodes in the chip was measured with an Agilent 4284A LCR meter (Agilent Technologies Inc., CA) connected to the chip through an Agilent 16048A BNC test

fixture. All of these instruments were connected to a computer through a GPIB interface. The impedance measurement process was controlled by LabView (National Instruments Corp., Austin, TX) virtual instruments. The impedance of interdigitated electrodes was measured at 52 frequencies logarithmically spaced between 100 Hz and 1 MHz with a 50 mV voltage excitation. Sinusoidal DEP signals were generated by an Agilent 33120A function generator.

2.4. On-chip cell capture

All the experiments were performed with the bioprocessor heated to 37 ± 0.2 $^{\circ}\text{C}$. The cells suspended in DMEM media were injected at an input flow rate of approximately 1.4 $\mu\text{L}/\text{min}$. The capture electrodes were excited with a 2 V_{pp} sinusoidal signal at 2 MHz. During injection, the flow rate in the incubation chamber was manually controlled to be between 4 to 10 $\mu\text{L}/\text{min}$. Flow rate control was performed using a flow controller based on the observed velocity of the cells in the channels. After the whole sample had been forced through the bioprocessor, the flow was stopped and the DEP capture electrodes were turned off. After pinching the tubes, the cells were incubated for a minimum of 24 h to form a confluent layer before the impedance measurement was started. To get the baseline impedance, a reference measurement was desired. Cells were injected into one device in a bioprocessor, where the other device received DMEM only. The device with DMEM media provided baseline impedance in the absence of endothelin-1.

3. Results and discussion

In this work, we have developed a novel bio-analytical system which can monitor cardiomyocyte hypertrophy in monolayers of adherent cells. This biosensor also provides convenient methods for cell concentration and patterning, which reduce the detection time and increase the sensitivity. The majority of existing techniques to monitor cardiac myocyte hypertrophy, *in vitro*, measure changes in (1) cellular protein content (by incorporation of radio-isotope labeled amino acids), (2) cardiomyocyte

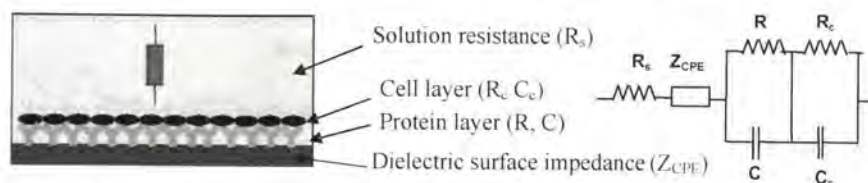


Fig. 3. Equivalent circuit for analysis. R_s is mainly due to the conductivity of the bulk solution and the wire connection, whereas the Z_{CPE} represents the dielectric properties of the electrode/electrolyte and surface morphological information. R and C are the resistance and the capacitance of the layer of the absorbed proteins. R_c and C_c are elements related to the cell layer.

morphology (by immunofluorescence labeling of myofilament proteins), or (3) kinase activity (such as focal adhesion kinase, FAK). The described cardiac myocyte impedance sensing system obviates these cumbersome experimental assays and provides a convenient and rapid *in vitro* method to measure cardiac myocyte hypertrophy.

3.1. The equivalent circuit of the impedance sensor

The impedance spectrum of this system can be represented by an equivalent circuit shown in Fig. 3. In this circuit, the resistor R_s is mainly due to the conductivity of the bulk solution and the wire connection, whereas the Z_{CPE} represents the dielectric properties of the electrode/electrolyte and surface morphological information. R and C are the resistance and the capacitance of the layer of the absorbed proteins. R_c and C_c are elements related to the cell layer. The overall impedance spectrum signal can be fitted by a mathematical model that was recently derived by Tlili et al. (2003) based on the electrochemical processes taking place at the interface. Fitting the model to impedance values in frequency spectrum over a large range makes the measurement more sensitive, i.e. an optimistic frequency can be chosen for the most sensitive impedance readings. Such fitted circuit model parameters essentially concentrates small changes of the impedance amplitude at each frequency point into larger changes which can be promising indicators with biophysical relevance.

The impedance spectra derived from equivalent circuits composed of ideal elements, like capacitors and resistors, do not fit the measured spectra in a satisfactory way because of the inhomogeneity of the surface. To overcome this problem, it is possible to replace the ideal elements by constant-phase elements (CPEs) Z_{CPE} (MacDonald, 1987; Omanovic and Metikos-Hukovic, 1995) which account for the non-linearities and the frequency of the elements by expressing the electrical impedance in terms:

$$Z_{CPE} = \frac{1}{Q_{CPE}(j\omega)^n}$$

where $j = \sqrt{-1}$. This simplest model can properly fit the measured data in the whole frequency spectrum and at all time points during incubation (Gomez-Sjoberg et al., 2005).

3.2. Characterization of the impedance sensor

The biochip was used to perform preliminary tests of the impedance technique with live cells incubated inside the chip. Suspensions of different concentrations of cardiac myocytes

were injected into the chip and incubated in an on-chip temperature around 37 °C for more than 24 h.

Fig. 4 compares the first and last impedance spectrum recorded in this experiment in two-dimensional representation. The open circle (○) represents the frequency dependent impedance spectrum before the cardiomyocyte monolayer was exposed to 100 nM endothelin-1 (ET-1) addition ($t=0$ h in Fig. 5), whereas the square (□) corresponds to the impedance spectrum of the same electrode after the cells were exposed to the 100 nM ET-1 and experienced the ET-1-induced cell morphology change ($t=4$ h in Fig. 5). As apparent from Fig. 4, the difference of total impedance amplitude between vital and hypertrophy cells passes a maximum at roughly 2 kHz indicating that impedance readings at this frequency are most sensitive to the associated changes in cell morphology.

3.3. On-chip detection of ET-induced cardiomyocyte hypertrophy

Since cardiac myocytes are a relatively high motility cell type, it is possible that the effects observed with the chemical agents are covered by cell motility rather than biochemical modulation. The inhibition of this motion is therefore required if biochemical modulation is to be confirmed. Cytochalasin-D has been used to successfully inhibit movement of cardiac myocytes, however, this compound (when used at high enough concentrations to inhibit movement) causes the cells to detach from the surface, resulting in a reduction of the measured impedance (Lo et al., 1993). Glucose deprivation has been shown to be a good way to reduce impedance fluctuations for confluent cells in monolayers over extended periods of time (Lo et al., 1993). Thus, in our

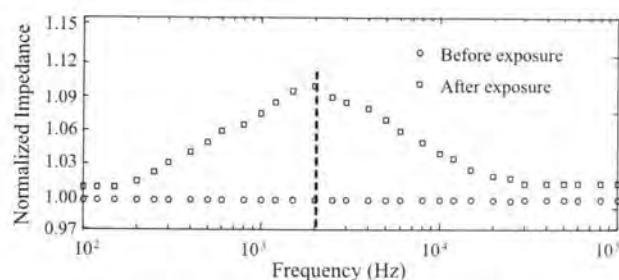


Fig. 4. Frequency spectrum of electrical impedance of gold-film electrodes covered with a confluent of cardiac myocyte cells before (○) and after (□) exposure to 100 nM endothelin-1 (ET-1) after 5 h. The dotted line indicates impedance values at a frequency of 2 kHz. The normalized impedance is defined as Z_{ET+}/Z_{ET-} . Z_{ET+} is the impedance after addition of ET-1, Z_{ET-} is the impedance before addition of ET-1.

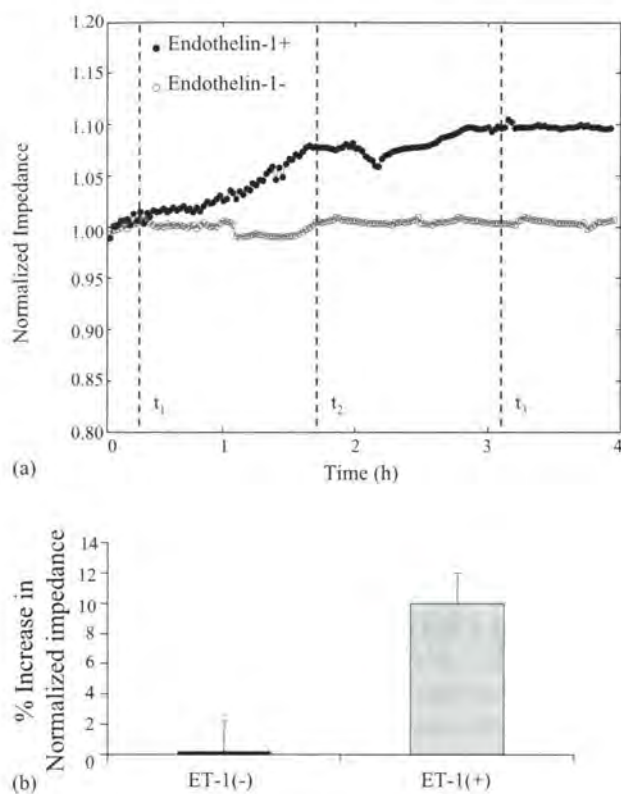


Fig. 5. Effects of ET on adhesion of cardiac myocytes to the extracellular matrix (ECM). Cardiac myocytes were cultured on the surface of laminin-coated wells manufactured especially for measurement of resistance. After glucose deprivation for 12 h, they were stimulated with ET-1 (endothelin-1) (nM). (a) Typical tracing representative of four replicate experiments. In all these experiments, initial resistance of cardiac myocytes was within the range of 3000–5000 Ω . To simplify the comparison, ordinate represents normalized impedance. (b) Comparison of percentage increase in normalized impedance at 4 h after addition of ET-1, ET(+), with control ET(–). Results shown are mean $\pm$ S.D. from four independent experiments.

experiment, the first impedance measurement was done about 12 h after the last media change of DMEM in incubation to decrease cell motility.

To assess the effects of ET-1 on the extent of cell-substrate adhesion, the changes in cell-substrate resistance were monitored after the addition of ET-1. We first monitored impedance values at frequency 2 KHz where a confluent monolayer of cardiomyocytes was exposed 100 nM ET-1. The extent of cell-substrate adhesion was expressed as “normalized” resistance, which was defined as a ratio of the resistance (Z_{ET+}) to the initial value before addition of ET-1 (Z_{ET-}). The addition of 100 nM ET-1-induced a rapid increase in impedance after an initial lag phase of around 0.3 h (Fig. 5a). Within 3 h the impedance magnitude approached the saturation value. To get a quantitative measure for the dynamics of ET-1-induced changes, we determined the time necessary for half-maximum reduction of the impedance at 2 KHz (t_{Z50}). The analysis showed that the mean value of 20 similar time courses was around 1.7 ± 0.2 h. ET-1-induced a significant increase in normalized resistance, indicating strengthening of the cell-substrate adhesion at 4 h after addition of ET-1 (Fig. 5b).

3.4. Cardiomyocyte hypertrophy detection with DEP concentration

To test the complete process of cell concentration and hypertrophy detection, cardiomyocytes, suspended in DMEM at concentrations of 4.6×10^3 and 5.3×10^4 mL^{-1} , were injected into the chip at a flow rate of approximately 1.4 $\mu\text{L}/\text{min}$ and incubated for at least 24 h at 37 $^\circ\text{C}$. To achieve a highly efficient concentration and capture process, the DEP forces in the capture stages of the bioprocessor had to be strong enough to counteract the drag forces exerted on the cells by the fluid around them. The DEP forces on cardiomyocytes suspended in DMEM were too weak to achieve an efficient concentration process at reasonable flow rates. However, cardiomyocytes experienced strong DEP forces at an excitation frequency of 2 MHz and the amplitude of 2 V_{pp} . With the right combination of medium conductivity, flow velocities, and DEP excitation voltage and frequency, the concentration efficiency was virtually 100%.

During injection of the sample with 5.3×10^4 mL^{-1} , the DEP electrodes were off so that the cells would not become concentrated in the incubation chamber. The other sample 4.6×10^3 mL^{-1} was injected with the DEP electrodes activated, which caused most of the cells in the samples to accumulate on the surface of microelectrodes. The actual number of cells collected was not known, but it was visually confirmed that only a very small fraction of the cells escaped the concentration process (no more than 5%). The frequencies chosen for plotting each curve, out of the 51 frequencies that were monitored, were those for which the relative impedance change was the largest at the frequency 2 KHz. Fig. 6a shows the effect of DEP concentration on the impedance spectrum with the addition of ET-1. In the absence of ET-1, the impedance spectrum of the concentration of 5.3×10^4 mL^{-1} did not change, as previously shown (Fig. 5a). The sample at concentration of 5.3×10^4 mL^{-1} injected without the DEP-based system active increased the impedance amplitude by 10% due to ET-1 addition. On the other hand, for the sample at relatively low concentration of 4.6×10^3 mL^{-1} injected with the DEP system active, an ET-1-induced increase in impedance amplitude of 18% was detectable in the impedance spectrum. These data demonstrated the utility of the DEP cell concentration method, as the ability to concentrate cells from a dilute sample on the impedance detection microelectrodes would further enhance the sensitivity of our biosensor.

3.5. Equivalent circuit parameter fitting

To correlate the variation of impedance to cardiomyocyte behavior, the fitting procedure with the previous equivalent circuit, defined without ET-1, is applied to impedance variation as a function of ET-1 concentration. Values of R_s , Z_{CPE} , R and C remain constant in the equivalent circuit while values of R_c and C_c components vary as a function of ET-1 concentration as shown in Fig. 6b. The error bars include the experimental error on impedance measurements and the discrepancy of experimental values related to the equivalent circuit model. As shown in Fig. 4, the cell layer resistance R_c changes from 4.52 to 8.43 $\Omega \text{ cm}^2$ due to cardiac myocyte hypertrophy, with an increase by 86.50% and

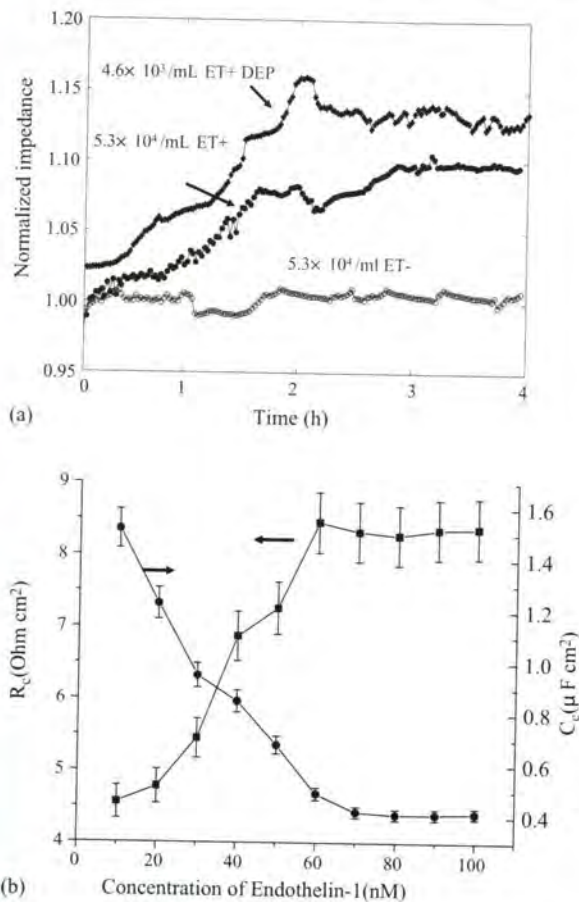


Fig. 6. (a) Normalized impedance of cardiac myocytes injected into the bioprocessor at various concentrations, with and without the DEP-based concentration system active. Values at $t=0$ are defined as 100%. (b) Impedance spectrum of electrodes with a confluent monolayer of cardiac myocytes for circuit model parameters R_c and C_c .

the capacitance C_c changes from 1.52 to 0.41 $\mu\text{F cm}^2$. Since R_c and C_c are elements related to cell layer (Fig. 3), the general evolution of R_c and C_c is indicative of the effect of ET-1 on cardiomyocyte morphology. A saturation effect is observed for both components for ET-1 concentrations higher than 60 nM which corresponds to the saturation effect on the experimental curve. The increase of R_c is related to the cell-substrate adhesion enhancement which can be used to study focal adhesion kinase (FAK) activity and the increase of C_c is more related to the cell size and cell-electrode gap change during the cardiomyocyte hypertrophy dynamic process. Combining with the immunochemical methods, the fitted circuit model parameters of

the impedance spectrum can be promising indicators to explore the various effects of ET-1 on the cardiomyocyte hypertrophy.

4. Summary and conclusions

In this work, a new cardiomyocyte-based impedance sensing system with the assistance of DEP cell concentration has been developed to monitor the dynamic process of endothelin-1-induced cardiomyocyte hypertrophy. The systems are fabricated on glass substrates utilizing microfabrication technologies. By integrating the DEP cell concentration system with the impedance sensing device, we are able to significantly increase the sensitivity of the impedance system, which reduces the time for detection by a significant factor. This novel and relatively simple biosensor has the potential to become a valuable high-throughput experimental approach in studying *in vitro* cardiomyocyte hypertrophy research.

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APPENDIX G

Real-Time Monitoring Primary Cardiomyocyte Adhesion Based on Electrochemical Impedance Spectroscopy and Electrical Cell–Substrate Impedance Sensing

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The cell–substrate distance is a direct indicator of cell adhesion to extracellular matrix which is indispensable in cell culture. A real-time monitoring approach can provide a detailed profile of cell adhesion, so that enables the detecting of adhesion-related cell behavior. In this work, we report a novel real-time impedance-based method to record the adhesion profile of cardiomyocyte, overcoming its inscrutability due to the primary culture. Micro-fabricated biosensors are applied in cardiomyocyte culture after characterizing the cell-free system. Cyclic frequency scanning data of cell-related impedance are generated and automatically fit into the equivalent circuit model, which is established using electrochemical impedance spectroscopy. The data are displayed as the alteration of normalized cell–substrate distance and the essential parameters for manual electric cell–substrate impedance sensing calibration of absolute distance. The time course displays a significant decline in the equivalent cell–substrate distance, from 155.8 to 60.2 nm in the first 20 h of cardiomyocyte culture. Furthermore, the cardiomyocytes cultured in long-term medium and short-term medium (ACCT) for 10 h exhibit distinct difference in adhesion rate as well as cell–substrate distance (72 vs 68 nm).

The ability to culture primary cardiomyocytes has resulted in the availability of a well-characterized in vitro system in which adhesion of cardiomyocytes to the extracellular matrix (ECM) is an important behavior. Research has proven that adhesion builds up the ECM-dependent signaling pathways for the cardiac hypertrophy,¹ apoptosis,² and migration, among other processes. Inside the cardiomyocytes, which are undergoing specific pathways, the formation or elimination of cell focal adhesions occurs as side tracks of signaling pathways. This leads to the strengthening or weakening of cell adhesion. In other words, the alteration in cell

adhesion is a cellular level response of cardiomyocytes to certain processes, which enables real-time monitoring of the cell adhesion to provide instant information regarding cell behavior. In particular, compared with qualitative optical observation, the acquisition of the change rate of cardiomyocyte adhesion will be a significant advancement in the field. Therefore, the need exists to develop a new biosensing system for real-time monitoring of cell adhesion, which is quantitative, convenient and noninvasive.

It has long been recognized that electric cell–substrate impedance sensing (ECIS) is valid and holds promise for monitoring the morphology, viability, and environmental change of the adherent cells.^{3,4} ECIS is a noninvasive and sensitive detecting technique for cell adhesion. In the last two decades, ECIS sensing has been employed to monitor many types of cells, including fibroblast, endothelial cells, etc.^{5–11} More recently, ECIS research turned to excitable cells, such as skeletal muscle cells¹² and cardiomyocytes.^{13,14} Generally, time courses of overall impedance during the biological processes were recorded in time intervals and attributed to various cell behaviors.

Despite the impressive advances in ECIS research with time courses of overall impedance, current and future studies strive to provide direct information of ECIS through advanced analyses. Giaever developed the data processing techniques catered to his ECIS results. In his method, paracellular current flow in the medium gap between disklike cells and electrodes is described

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in the infinite element model,¹⁵ where the cell body is assumed exhibiting known and stable capacitance. It has been reported that this advanced ECIS method is capable of detecting vertical motion in the order of 1 nm⁴. Lo¹⁶ further developed an advanced ECIS method with the cell model constructed with a flat rectangular box and a half disk on each end, enabling the advanced ECIS to cells that are not so symmetric as that of Giaever's. However, unlike endothelial cells or fibroblasts in the reported ECIS cases that show known and constant electrical properties due to the reliable commercialized cell lines, cardiomyocytes are primarily cultured, which means membrane impedance of cardiomyocytes are unknown and various. Furthermore, Lo's model has two adjustable parameters, which are to be determined in the curve fitting. The fitting methodology is developed from a nonlinear optimization in which an initial guess is one of most important criteria. The iterative process can converge too slowly or even fail without a reasonable initial guess. Unfortunately, these adjustable parameters are absent in the research on cardiomyocytes whereas those of endothelial cells and fibroblasts are reported and then utilized in the ECIS for these types of cells. These factors are essentially the bottleneck of ECIS application to cardiomyocyte research.

In this research, electrochemical impedance spectroscopy (EIS), a powerful approach to the study of electrolyte-electrode interface, is invoked to the impedance sensing and analysis. Frequency scanning is applied in electrical measurements instead of recording impedance data at a single frequency. Impedance data are used to determine the components of equivalent circuit models based on EIS. We show that, as cardiomyocytes are cultured on the biosensors, the resistance of the cell-substrate gap exhibits the increscent tendency. This process can be manually calibrated with ECIS and translated into a decreasing time course of an equivalent cell-substrate gap. Moreover, the comparison of cardiomyocytes cultured in long-term medium (LTM) and ACCT demonstrates the distinction of cell adhesion in different media. These results support cardiomyocyte adhesion processes observed in biological experiments and validate our impedance sensing and analysis system for real-time monitoring of cardiomyocytes.

EXPERIMENTAL SECTION

Fabrication. The fabrication of the biosensing chips was conducted in a microfabrication laboratory. A glass slide was chosen as the substrate material because it could minimize the substrate capacitance and decrease the measurement noise. The electrode voids of the chips were patterned on the slides with photoresist Shipley 1813 in photolithography on the MA6 aligner. Effective adhesion of the metal electrodes to the glass substrate is required; therefore, a chromium layer (125 Å) was first deposited in thermal evaporation, followed by the deposition of the major electrode components with gold (375 Å) (Kurt J. Lesker Co.). The electrodes were subsequently defined in a liftoff process. SiO₂ and SiN_x (Kurt J. Lesker Co.) were deposited with magnetic sputtering to form an insulating layer 1200 Å thick. The metal electrodes were exposed in reactive ion etching process. Figure 1 shows the configuration of the biosensing chip.

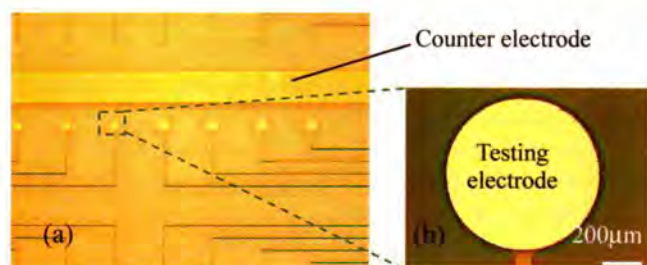


Figure 1. (a) Top view of the bioimpedance sensor. (b) Close view of the testing electrode fabricated with liftoff methods (Cr/Au: 125 Å/375 Å). Chromium was used to increase the adhesion of the gold layer to the glass substrate. A laminin (an ECM protein) layer was coated before cardiomyocytes were injected.

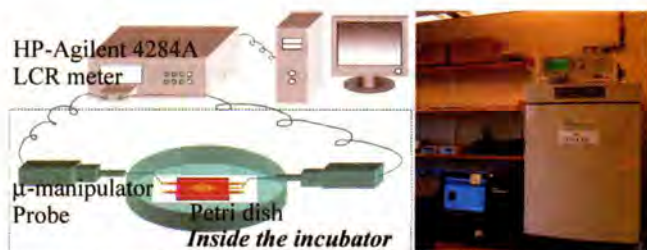


Figure 2. Schematic diagram and photo of the impedance measurement system. The part surrounded by dash lines is inside the incubator (37 °C, 5% CO₂).

Testing Systems. The schematic diagram and photo of our impedance-sensing system is depicted in Figure 2. The biosensing chip was maintained in the incubator at 5% CO₂ and 37 °C throughout the electrical measurement to avoid any fluctuations in the testing environment. Inside the incubator, the biosensing chip was mounted to a homemade silicone chamber designed for cell culture. The electrode pads were connected to micromanipulators, which transferred signals through an Agilent 16048A BNC test fixture to the outside of the chamber. The impedance of the electrodes in the chip was measured with an Agilent 4284A LCR meter (Agilent Technologies Inc.). For automatic measurement and data logging, the LCR meter was connected to a computer through a GPIB interface. The impedance measurement process was controlled by LabView (National Instruments Corp., Austin, TX) virtual instruments.

Cardiomyocyte Isolation. Left ventricular myocytes were isolated from male Wistar rats according to a previously established protocol.¹⁷ The left ventricle was separated, minced, and gently agitated allowing the myocytes to disperse in KB solution (85 mmol/L KOH, 30 mmol/L KCl, 30 mmol/L KH₂PO₄, 3 mmol/L MgSO₄, 0.5 mmol/L EGTA, 10 mmol/L HEPES, 50 mmol/L L-glutamic acid, 20 mmol/L taurine, 10 mmol/L 2,3-butanedione monoxime, and 10 mmol/L glucose). The cardiomyocyte suspension was subsequently filtered through a nylon mesh, resulting in a typical yield of >90% rod-shaped cells. The isolated cells were then washed with DMEM, with the addition of ascorbic acid (100 μM), and were ready for plating onto microelectrodes. The initial cardiomyocyte adhesion monitoring was performed to the cells in DMEM for the first 20 h following the initiation of the culture.

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Electrical Measurement of Cardiomyocyte Adhesion. A laminin (Becton-Dickinson), the ECM protein, ACCT medium suspension was injected onto the testing electrode and maintained in a still incubator for at least 30 min until a laminin layer formed. The characterization of the laminin layer formed on the testing electrode was then performed with 80 frequencies logarithmically spaced between 20 Hz and 200 kHz, with a 5-mV voltage excitation. The frequency scanning was completed in 1 min. After characterization, the laminin suspension was removed, and the cardiomyocyte suspension was injected in the silicone chamber. The electrical monitoring of cell adhesion was initiated immediately after the injection. The cyclic frequency scanning was applied to the testing system every 10 min in a 20-h cell culture period. The impedance data were recorded as real and imaginary components accompanied by the testing frequency in a txt file, which was convenient for the following data processing and analysis.

Cardiomyocyte Culture in Short-Term Medium and Long-Term Medium. Two different types of media were tested in this research, including (1) short-term medium (ACCT) (0.2 wt % albumin, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine in low glucose DMEM); and (2) long-term medium (2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 μ M BrDU, and 7 vol % heat-inactivated fetal bovine serum in low glucose DMEM). After 1-h initial culture in DMEM, a comparison of cardiomyocyte adhesion in different media was performed using cardiomyocyte culture samples. The cyclic frequency scanning protocol was applied to the testing system in the following 10 h of cell culture. The same settings used in monitoring the initial cardiomyocytes adhesion were replicated.

RESULTS AND DISCUSSION

In this work, the time course of cell-substrate distance was extracted from the impedance data processed with the EIS and ECIS, which could be direct monitoring of cardiomyocyte adhesion to ECM. Equivalent circuit models for protein-coating and cell layers were built and modified according to the characteristics of impedance spectra. In this way, the mode of alternating current flow could be precisely described, which could further reveal the nature of cardiomyocyte adhesion. Of all elements in the equivalent circuit model, the resistance of the medium layer in cell-substrate gap was the most straightforward quantitative indicators describing the extent of adhesion. The dynamics of the adhesion process could be recorded by connecting the parameters of equivalent circuit determined in the cyclic frequency scanning. With manual ECIS calibration, the time course of equivalent cell-substrate distance was calculated for the cardiomyocytes under the following conditions: (1) the initial culture in DMEM; (2) the culture after the medium changed to ACCT; and (3) the culture after the medium change to LTM.

Characterization of Protein-Coated Gold Electrodes. Protein coating to the microelectrode surface is necessary for cardiomyocyte culture. However, the protein coating layer will cause an extra addition to impedance by blocking the excitation signal from the gold electrodes. It is necessary to separate the impedance components induced by the protein coating layer.

The impedance-sensing system without cells can be represented by the equivalent circuit model shown in Figure 3a. Here, the dielectric characteristics of the protein layer (laminin) are considered an ideal capacitor (C_p) on the electrode surface.

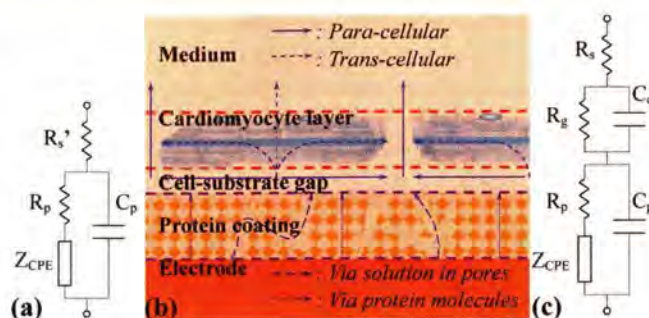


Figure 3. (a) Equivalent circuit model of system impedance without cells. R_s is the resistance of bulk solution and the wire connection. The capacitance of laminin layer is represented by C_p . Z_{CPE} and R_p are used to describe nonideal capacitive response of the uncovered electrode surface and the resistance in the pores of laminin layer. (b) Current flow patterns on the testing electrode. There are two modes of current in protein coating layer: one travels via solution in the pores among protein molecules; the other travels via protein molecules. There are also two modes of current in cardiomyocyte layer: Paracellular mode represents how the current travels in solution around cells; transcellular mode represents how the current travels through the cell body. (c) Simplified equivalent circuit model of impedance of the cell-covered electrode (Z_c). The impedance response to paracellular or transcellular current is described with resistors or capacitors respectively. Especially, R_g is the resistance of medium layer in the cell-substrate gap. When cardiomyocytes begin to adhere to ECM protein, the descent of the cell-substrate gap will result in the increase of R_g .

However, since laminin are macromolecules, which are ~ 900 kDa in molecular mass and ~ 10 nm in length,¹⁸ it is difficult to achieve the complete and compact coverage of laminin over the electrode surface. Research does demonstrate the pores exist among the protein macromolecules.¹⁹ Solution can enter these poles and reach the gold electrode surface (Figure 3b), which can induce nonideal capacitance and resistance. Therefore, a constant-phase element (CPE) is used to account for the nonlinearities of frequency-related electrical double layer impedance ($Z_{CPE} = [Q_{CPE}(j\omega)^n]^{-1}$, where j and ω is the imaginary unit and angular frequency, respectively) on the naked gold surface.^{20,21} The resistor R_p is used to represent the pore resistance in the laminin layer. Both the current flows via protein molecules and via pores in protein coating layer travel in the bulk medium represented with a resistor (R_s).

The equivalent circuit model was fit to the 3D impedance response plot of the impedance-sensing system, as shown in Figure 4. To determine the element parameters of the impedance-sensing system, a complex nonlinear least-squares (CNLS) curve fitting was performed to eq 1

$$Z_{nt} = R'_s + \frac{1}{j\omega C_p + \frac{1}{R_p + \frac{1}{Q_{CPE}(j\omega)^n}}} \quad (1)$$

derived from the equivalent circuit model in Figure 3a: where Z_{nt} is the theoretical value of specific impedance of cell-free system (Z_n). The P weighting²² was added into the objective function of

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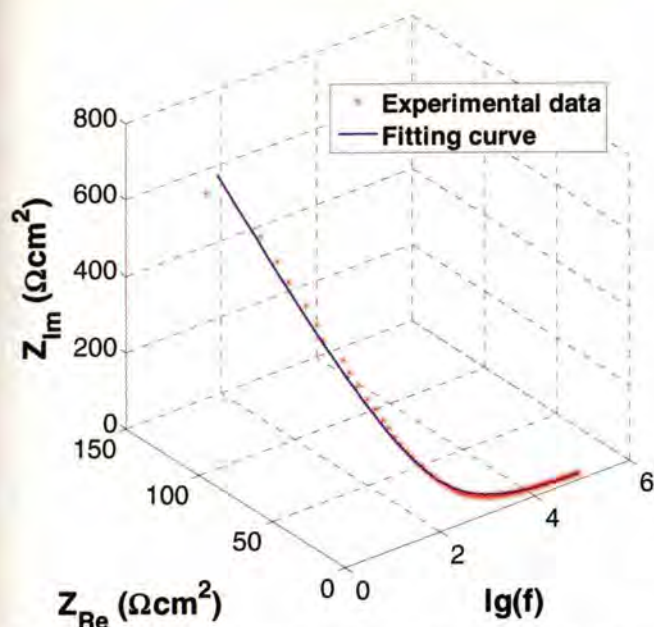


Figure 4. Characterization of cell-free impedance sensing system (Z_n) with CNLS curve fitting to eq 1 with the P weighting in the objective function. The curve fitting was performed with a program developed from optimizing function toolbox of MATLAB. According to the convention of the electrochemical society, $Z = Z_{Re} - jZ_{Im}$.

CNLS curve fitting (S) to ensure the validity in case the fitting to the real or imaginary component of impedance is unbalanced:

$$S = \sum_{i=1}^k \left\{ \left[1 - \frac{Z_{nt,Re}(\omega_k)}{Z_{n,k,Re}} \right]^2 + \left[1 - \frac{Z_{nt,Im}(\omega_k)}{Z_{n,k,Im}} \right]^2 \right\} \quad (2)$$

Despite the existing curve-fitting software, for example, LEVM, a CNLS curve-fitting program was written based on the optimizing toolbox of MATLAB. This allowed for a convenient means for the impedance data obtained in LabView virtual instruments and the following calculations and plotting. The element parameters are simultaneously determined: $R'_c = 4.39 \pm 0.05 \Omega \cdot \text{cm}^2$, $C_p = 3.03 \pm 0.12 \mu\text{F}/\text{cm}^2$, $R_p = 13.98 \pm 0.31 \Omega \cdot \text{cm}^2$, $Q_{CPE} = 16.42 \pm 0.33 \mu\text{F}/\text{cm}^2$, and $n = 0.827 \pm 0.003$.

Monitoring the Initial Adhesion of Cardiomyocyte. The monitoring technique of cardiomyocyte adhesion was developed based on the cyclic frequency scanning of impedance and calculation of cell-substrate distance, which began with separating the cell-related impedance (Z_r) from the overall (Z_c). According to the experiment protocol, the DMEM-containing laminin was drained and replaced with cardiomyocyte-suspended DMEM. Thus, the total impedance can be considered as the combination of Z_r and Z_n with the exception for R'_s . Therefore, Z_r values could be calculated ($Z_r = Z_c - (Z_n - R'_s)$).

To acquire more electrical parameters from the impedance measurement, we build the cardiomyocyte layer model on an electrode based on EIS, yet most assumptions in ECIS are

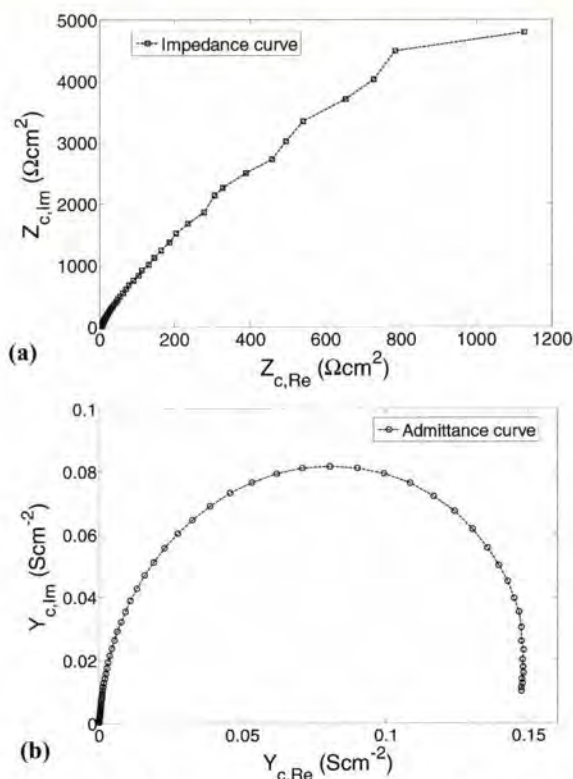


Figure 5. Typical Nyquist plots of Z_r and Y_r . These plots were taken automatically in the cyclic frequency scanning. The Nyquist plots of impedance and admittance are close to 1/6 of a circle and semicircle, respectively. Since the geometry of the admittance curve is more ideal than that of the impedance curve, it is easier to obtain the initial guess from the admittance curve than from the impedance curve.

maintained for the coherence of these two methods. As the testing voltage is applied to cells cultured on electrodes, the ac current flow is divided into the transcellular component and paracellular component, which travels via cells and via cell-substrate gap, respectively. Thus, in our equivalent circuit model of Z_r (Figure 3c), C_c is used to represent the reactance of cells and R_g is used to describe the resistance of the thin medium layer in the cell-substrate gap. R_s is the resistance of bulk medium body. Theoretical equation of Z_r derived from this model is

$$Z_{rt} = R_s + \frac{1}{j\omega C_c + (1/R_g)} \quad (3)$$

where Z_{rt} is the theoretical value of Z_r . The CNLS curve fitting with P weighting was employed here to determine the component values.

To achieve the automation in series of CNLS curve fitting, its initial guess is given by analyzing the characteristics of the impedance response in a Nyquist plot. When reversed, the impedance response data can be converted to admittance response ($Y_r = Z_r^{-1}$). The Nyquist plot of admittance response is a semicircle in the first quadrant that can be expressed as

$$(Y_{r,Re} - d - r)^2 + Y_{r,Im}^2 = (r/2)^2 \quad (4)$$

where d and r are the intercept and radius of the admittance semicircle. The electrical parameters, such as R_s and R_g , can be

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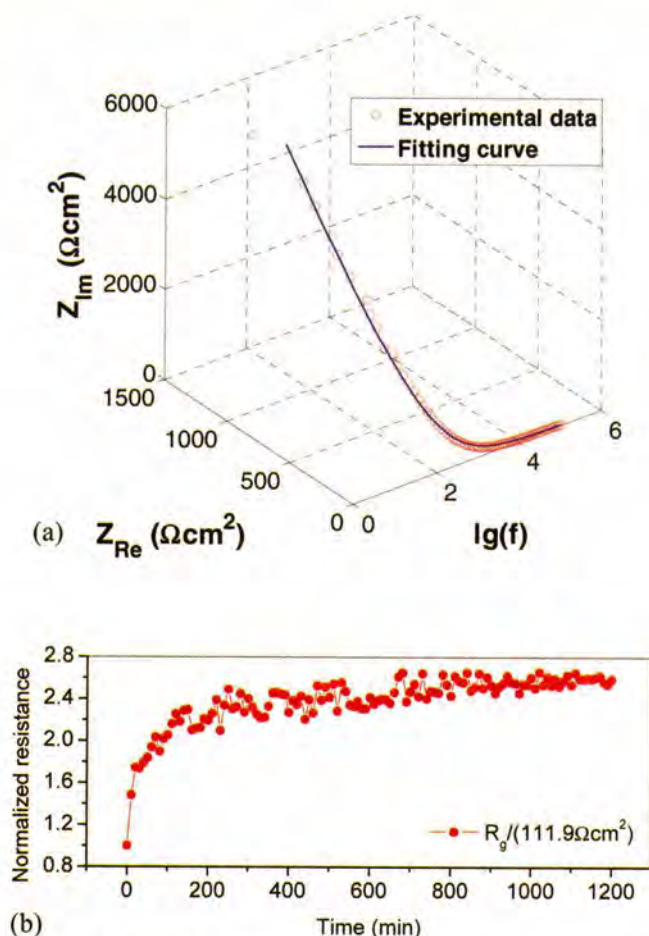


Figure 6. (a) CNLS curve fitting of the cell-related impedance to the EIS model (eq 3) with the P weighting in the objective function (eq 4). The related electrical parameters such as R_g and C_c are determined. (b) Alteration of normalized R_g in the first 20 h of cardiomyocyte culture. The initial value ($R_{g0} = 111.9 \Omega \cdot \text{cm}^2$) is set as normalizing units. R_g rapidly increased within the first hour and slowly reached its saturation within 20 h.

obtained by calculating eq 5 and eq 6, of which d and r are major parameters:

$$R_s = \frac{-d - r + \sqrt{9d^2 + 18dr + r^2}}{2d^2 + 4dr} \quad (5)$$

$$R_g = \frac{(r + d)R_s^2 - R_s^2}{2 - (r + d)R_s^2} \quad (6)$$

And C_c can also be calculated through eq 3. The reason for using admittance response instead of impedance response is the Nyquist plot of admittance is more orbicular than that of impedance, which is close to 1/6 of the circle (Figure 5).

The electrical parameters acquired in the Nyquist plots of Y_r are utilized as the initial guess of the following CNLS curve fitting (Figure 6a). Every loop in the cyclic frequency scanning goes through the EIS data processing to determine R_g , R_s , and C_c values.

The normalized time course of equivalent cell-substrate distance is defined based on normalizing the time course of R_g to its initial value. Its rationality is ensured by the following discussion. In our model, R_g describes the resistance to the

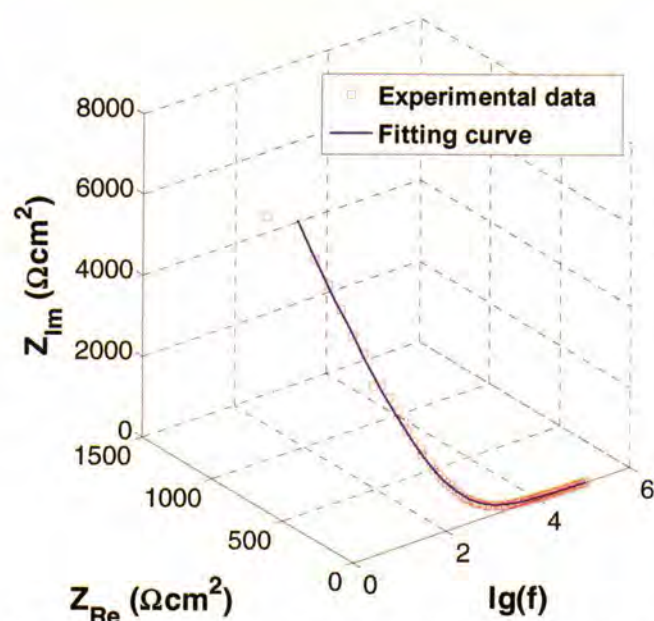


Figure 7. Calibration of the initial equivalent cell-substrate distance via the CNLS curve fitting of the total impedance (Z_c) to the ECIS model (eq 8) with the P weighting in the objective function.

paracellular current traveling horizontally through the cell-substrate gap. The cell-substrate distance of living cells is in the range of 50 nm,^{23,24} a distance much smaller than the dimension of cardiomyocytes. Thus, R_g values are highly related to gap dimensions and ion conductivity. However, the ion concentration fluctuation is excluded here because molecular dynamics simulations have demonstrated that fluctuation in ion or solvent particle density occurs only in the interface layer adjacent to regions less than 1 nm thick.²⁵ Since the existence of R_g is owing to the resistivity of the conductive solution, which obeys Ohm's law, it can be assumed that normalized R_g is inversely proportional to the normalized equivalent cell-substrate distance.

R_g (or reciprocal equivalent cell-substrate distance) change was drawn in the time course after normalization to the initial values (Figure 6b). The normalized resistance rapidly rose to ~184% within the first hour of culture and slowly approached the saturation point of 260% at the end of the first 20 h of culture. Given cardiomyocytes show minor morphological change prior to the dedifferentiation, the change in the cell-substrate gap is mainly distributed by cell adhesion. The cell adhesion initially rose exponentially, reached a plateau, and in the subsequent hours reached saturation.

ECIS Calibration of Cell-Substrate Distance. As described above, EIS data processing of the cell-related impedance can provide the electrical parameters of the cardiomyocyte layer. If the impedance through the body of the cell is considered the capacitive reactance of the cell membrane, all parameters are prepared to calculate absolute cell-substrate distance via advanced analysis of ECIS. However, since no reasonable initial guess is available for the adjustable parameters, the difficulties

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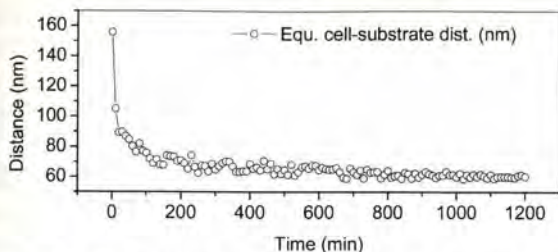


Figure 8. Time course of equivalent cell-substrate distance obtained by calculating the reciprocal of normalized R_g curve followed by the manual calibration of the initial distance with ECIS model. The cell-substrate distance decreases from 155.8 to 60.2 nm within 20 h. The alteration of cell-substrate distance directly describes the adhesion status of cardiomyocytes in primary culture.

in the iteration process will restrict its application in processing isolated data at a single moment. In this research, the advanced ECIS is introduced to calibrate the absolute cell-substrate distance with the manual selection of the initial guess. Combined with the normalized tendency obtained above, the time course of absolute cell-substrate distance can be utilized to record the cardiomyocyte adhesion.

Lo's advanced analysis of ECIS¹⁶ is called in for the calibration, where the geometry of isolated cardiomyocyte is described as a flat rectangular ($L \times W$) box and a half disk ($r_c = W/2$) on each end. In this method, specific impedances of cell-covered (Z_c) and cell-free (Z_n) electrodes are calculated by multiplying the measured impedance by the electrode area. Since the cell membrane principally shows a dielectric nature, the impedance through the cell body is approximately the reactance of C_c in the EIS model, which can be expressed as $Z_m = -j/(\omega C_c)$. One of the two adjustable parameters in Lo's model is the morphological parameter (α) of cell-substrate distance is set as

$$\alpha = \frac{W}{2} \sqrt{\frac{\rho}{h}} \quad (7)$$

where the medium resistivity is ρ and h is the equivalent cell-substrate distance. The other one, R_b , is specific junctional resistances between adjacent cells.

The analytical solution of this method describes the combination of the known impedance data Z_n and Z_m , and two adjustable parameters, α and R_b equals Z_c .¹⁶

$$\frac{1}{Z_c} = \frac{1}{Z_n + Z_m} \left[1 + \left(\frac{LW}{LW + \frac{\pi W^2}{4}} \right) \frac{\frac{2Z_m}{Z_n}}{\gamma W \coth\left(\frac{\gamma W}{2}\right) + 2.328R_b\left(\frac{1}{Z_n} + \frac{1}{Z_m}\right)} + \left(\frac{\frac{\pi W^2}{4}}{LW + \frac{\pi W^2}{4}} \right) \frac{\frac{2Z_m}{Z_n}}{\frac{\gamma W I_0(\gamma W/2)}{2 I_1(\gamma W/2)} + 1.164R_b\left(\frac{1}{Z_n} + \frac{1}{Z_m}\right)} \right] \quad (8)$$

where $\gamma = [W(Z_n^{-1} + Z_m^{-1})/2]^{-1}$.

The Z_c values were fitted to eq 8 in CNLS curve fitting with the P weighting similar to eq 2. The best fit of ECIS (Figure 7)

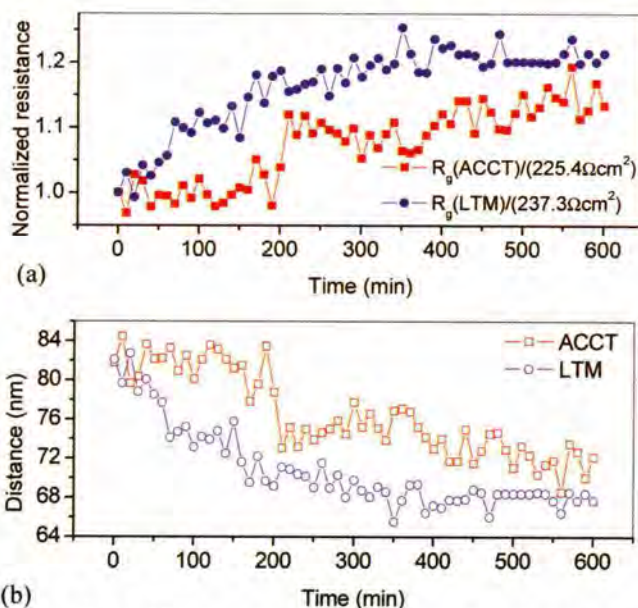


Figure 9. (a) Alteration of normalized R_g in the first 10 h of culture since changing the medium to LTM and ACCT after the initial culture in DMEM for 1 h. The normalizing units are the initial values of R_g in both samples, $R_{g0} = 225.4 \Omega \cdot \text{cm}^2$ for the ACCT sample; $R_{g0} = 237.3 \Omega \cdot \text{cm}^2$ for the LTM sample. (b) The time courses of equivalent cell-substrate distance are obtained by calculating the reciprocal of normalized R_g curve followed by the manual calibration of initial distance with the ECIS model. The initial distances are almost equivalent, cardiomyocytes cultured in LTM exhibit better adhesion than those in ACCT in terms of a smaller cell-substrate distance and a faster adhesion rate. The difference of adhesion in LTM and ACCT is owing to the existence of the growth factors in LTM.

resulted in $\alpha = 6.798 \Omega^{1/2} \cdot \text{cm}$ and $R_b = 0.835 \Omega \cdot \text{cm}^2$. Given the resistivity of DMEM at 37 °C is $72 \Omega \cdot \text{cm}$, the corresponding equivalent cell-substrate distance at the beginning of the initial culture was 155.8 nm, derived via the calculation of eq 7. Once its initial value was calibrated, the whole time course of cell-substrate distance was easily calculated based on the assumption of an inverse proportional relationship between R_g and cell-substrate distance. Figure 8 depicts the alteration of equivalent cell-substrate distance in the initial stage of cell culture, which matches the known cell adhesion process of cardiomyocytes. After 20 h of culture, cardiomyocytes reach a height of 60.2 nm from the laminin substrate, which is consistent with published reports.

Comparison of Cardiomyocyte Culture in ACCT and LTM.

The fresh isolated cardiomyocytes were first cultured in DMEM for ~ 1 h, allowing the initial adhesion of cardiomyocyte to rapidly occur. Afterward, the culture medium was changed to ACCT or LTM. In the primary culture of cardiomyocytes, researchers often use different culture media for specific cell requirements.

As the result of the experiment, the following 10-h culture in ACCT or LTM demonstrated different influences on cardiomyocyte adhesion, as shown in Figure 9a. The increment of normalized R_g of cardiomyocyte culture in LTM is $\sim 20\%$ while that of ACCT is $\sim 15\%$. Generally speaking, the major difference between ACCT and LTM in our experiment is that LTM carries fetal bovine serum, absent in ACCT. Fetal bovine serum contains multiple growth factors, including insulin-like growth factor I (IGF-I) as a

typical constituent.²⁶ Targeted to the IGF-I receptor, IGF-I can be used to promote physiologic hypertrophy,²⁷ accompanied by the activation of focal adhesion kinase²⁸ in the signaling pathways. The activation of focal adhesion kinase directly indicates the strengthening of cell adhesion.

Calibrated with the advanced ECIS mentioned above, Figure 9b illustrates the alteration of equivalent cell-substrate distance. Despite the fact that initial distances are very close, the LTM sample is more tightly adhered to the substrate than the ACCT sample (68 vs 72 nm). In addition, a comparison of decreasing rate also shows the LTM sample adheres faster than the ACCT sample, indicating LTM promotes cell adhesion more than that of ACCT.

CONCLUSION

In this work, a new sensing and analyzing system is established for the impedance signals in real-time monitoring of cardiomyocyte adhesion to ECM. Though impedance-sensing techniques have been successful with several kinds of biological cells, advanced analysis of pervious impedance sensing is not applicable to cardiomyocyte due to its unknown and variable electrical parameters. The new technique is founded on EIS and experimental evidence, using an equivalent circuit model to describe current

pattern on the cell-substrate interface. The new method is convenient for real-time recording the normalized alteration of cell-substrate distance because it enables an automatic data processing with CNLS curve fitting. Simultaneously, specific impedance of the cardiomyocyte layer is worked out for manually calculating the equivalent cell-substrate distance based on ECIS methodology. For the first time, the equivalent cell-substrate distance was recorded in real time and calibrated for the cardiomyocyte culture in time courses. The dynamics processes of initial adhesion and comparison of adhesion rates in ACCT and LTM were recorded and proved the validity of our method of real-time monitoring of cardiomyocyte biological processes.

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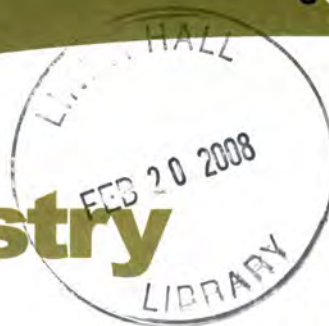
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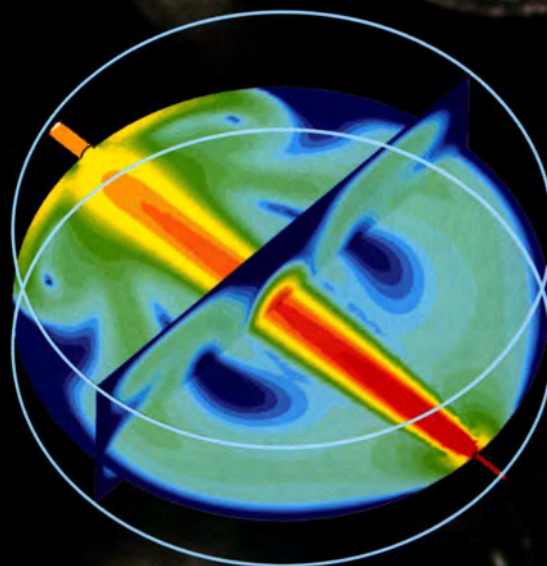
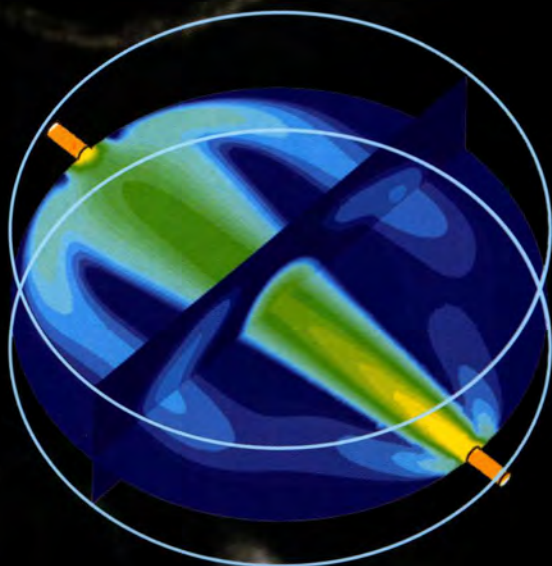
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