

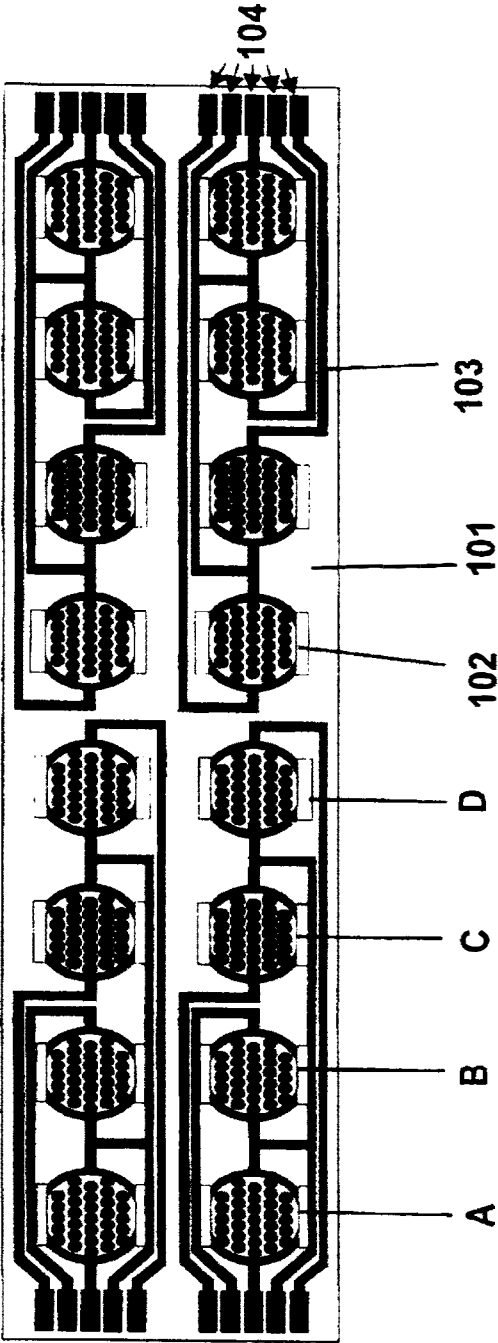


FIG. 1A

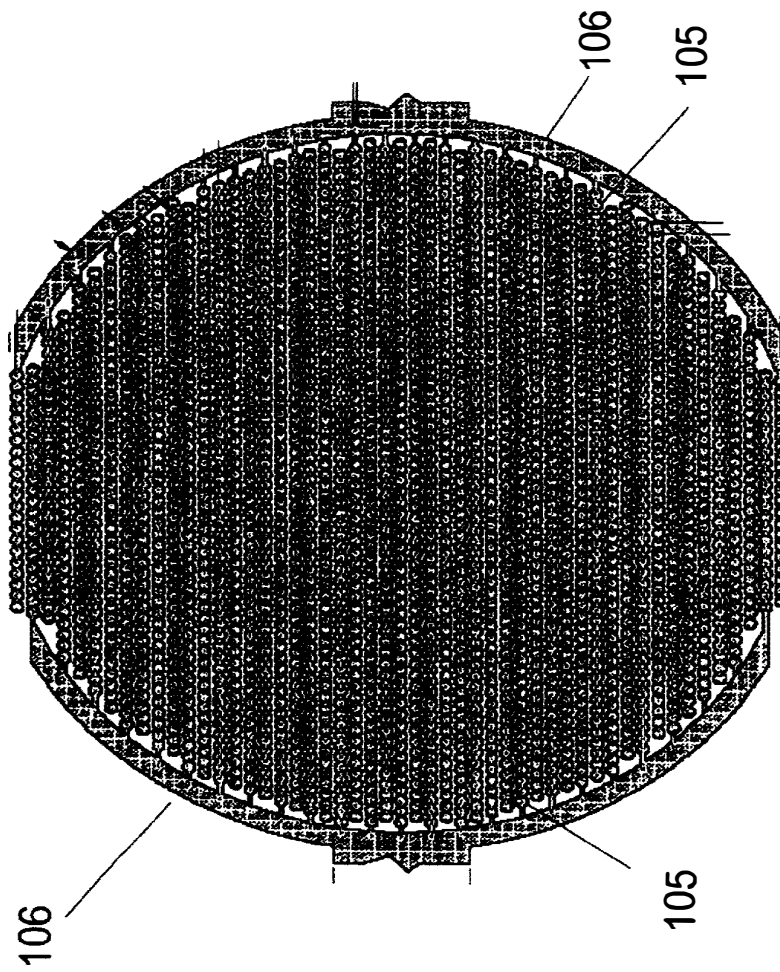


FIG. 1B

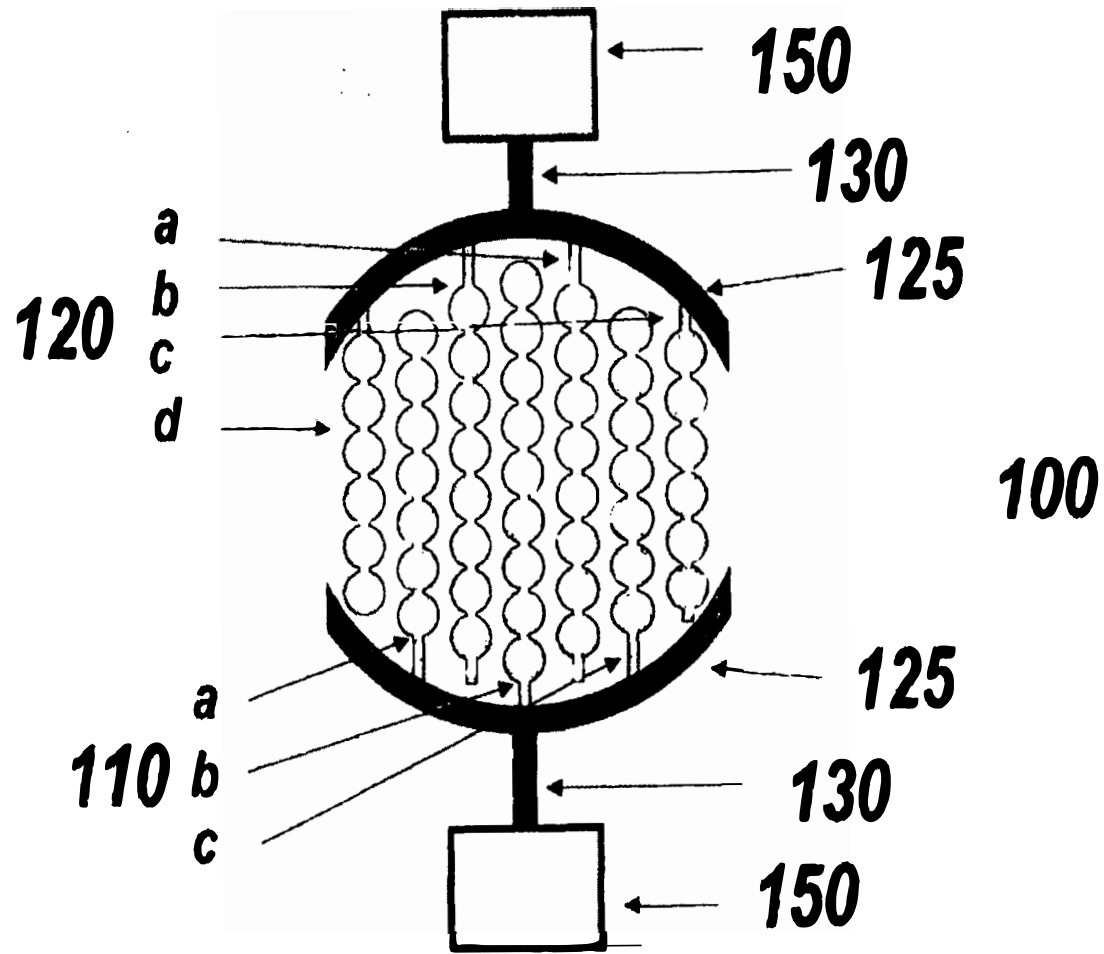
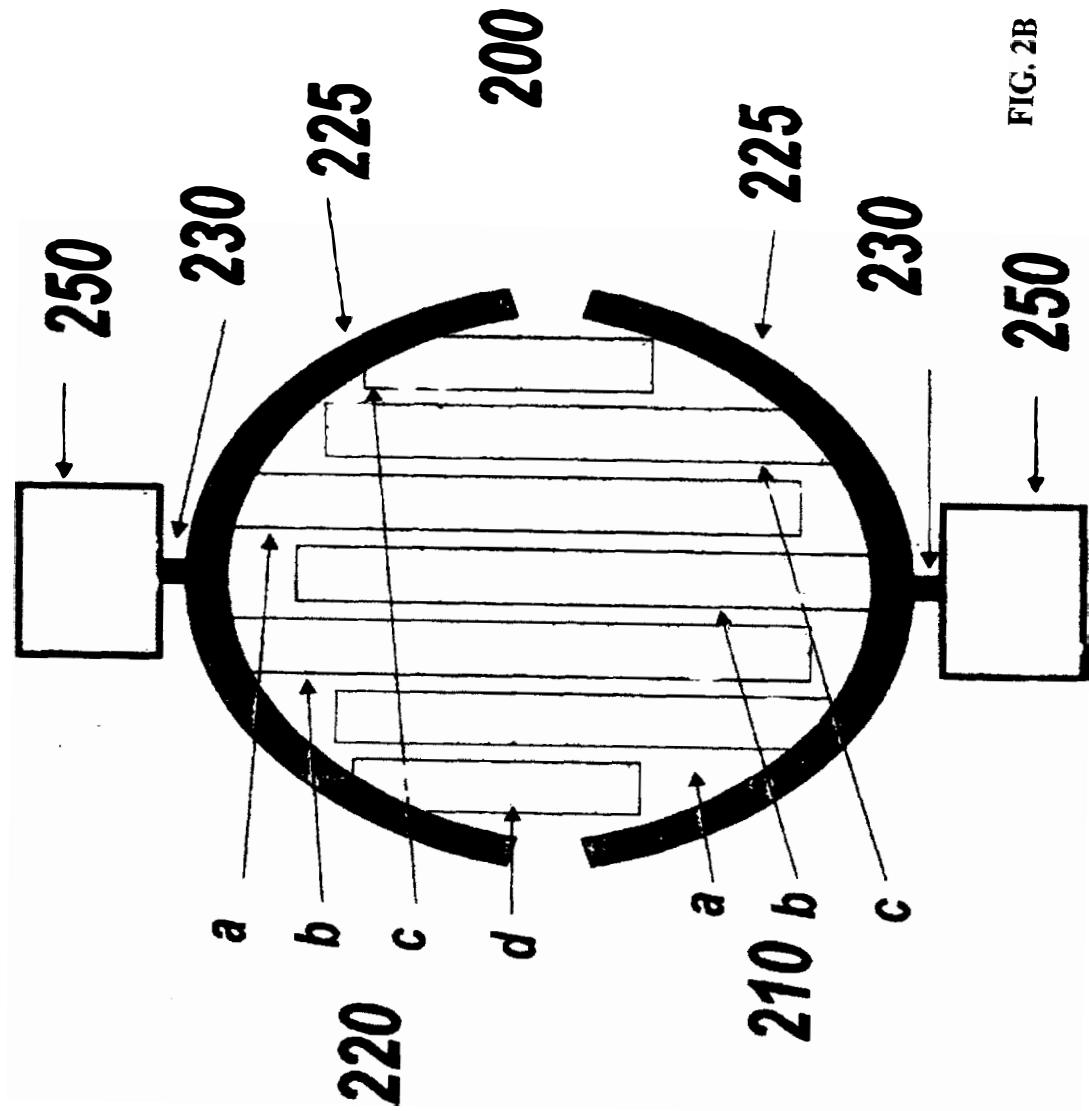
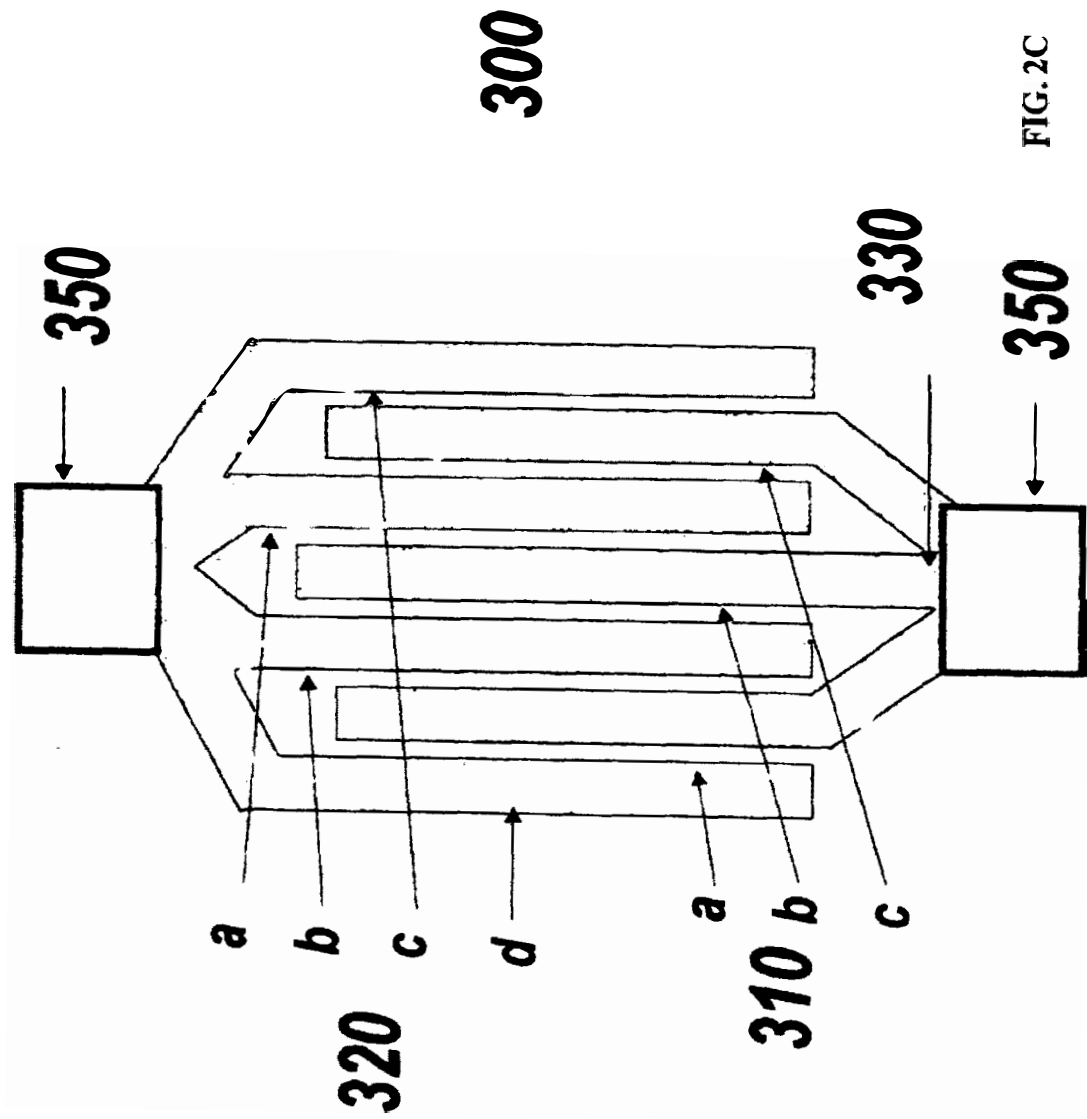
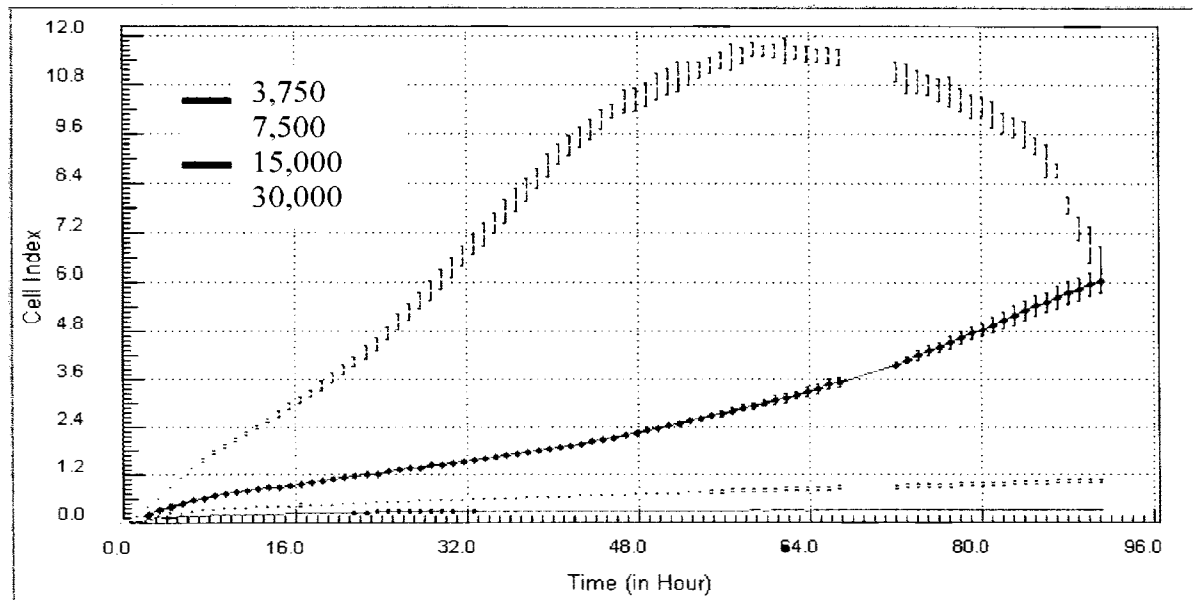


FIG. 2A





6 / 42

FIG. 3**Titration of ES-derived cardiomyocytes using the ACEA RT-CES system**

7 / 42

FIG. 4 Treatment of ES-derived cardiomyocytes with a cytotoxic compound of Sodium dichromate dehydrate.

FIG. 4A

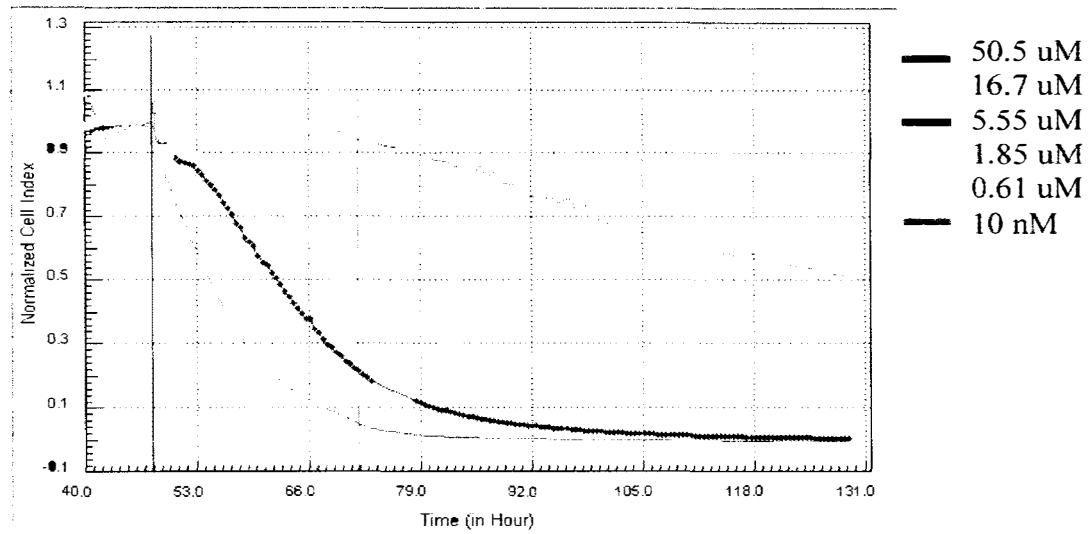
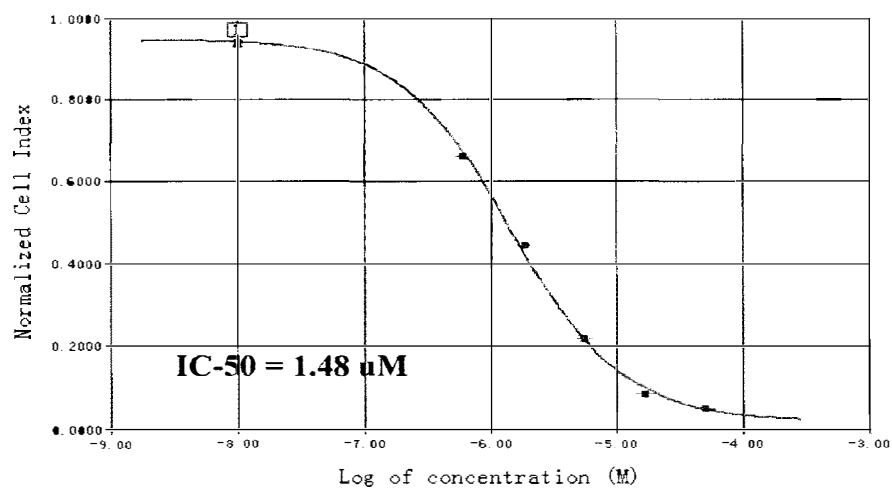


FIG. 4B



8 / 42

FIG. 5. Detection of morphological changes associated with ES-derived cardiomyocytes induced by isoproterenol, a β_2 adrenergic receptor agonist, using the RT-CES system

Fig. 5A.

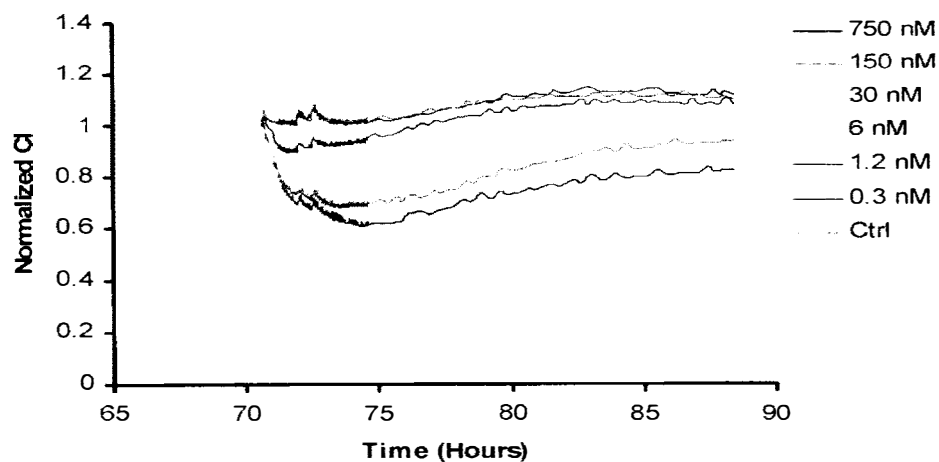
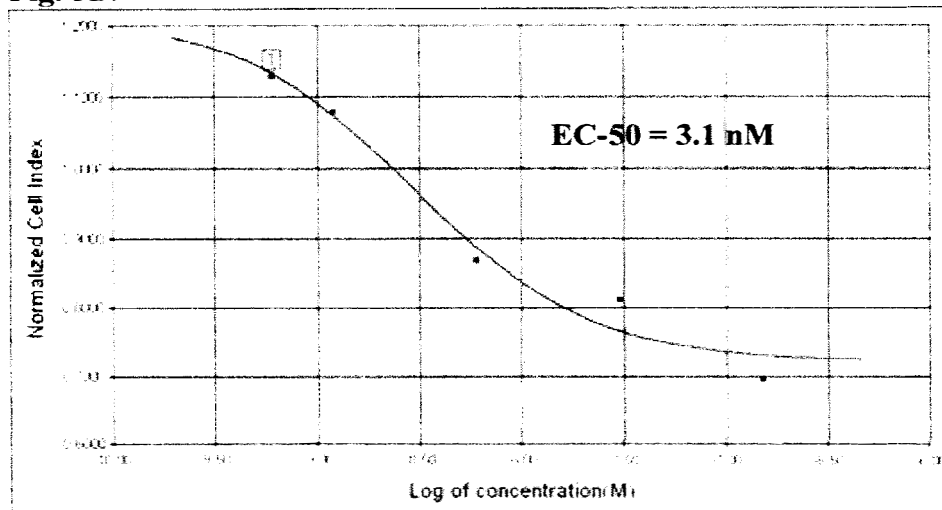
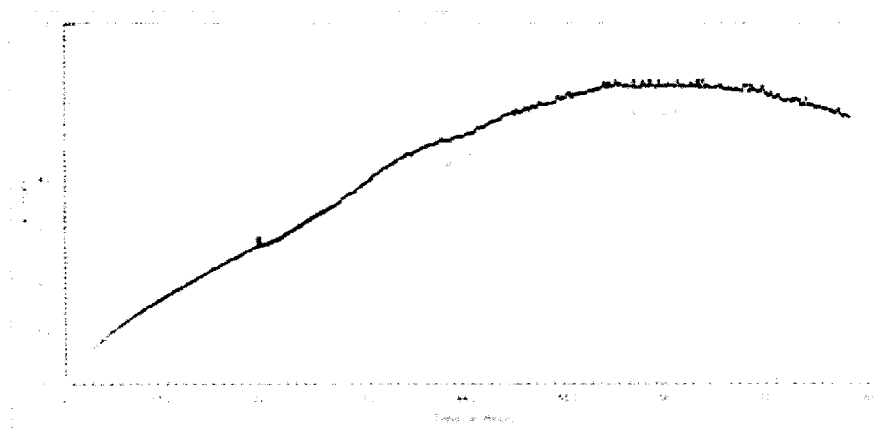


Fig. 5B.



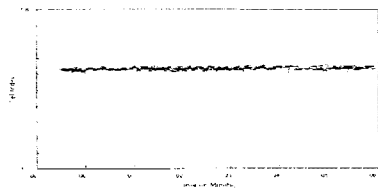
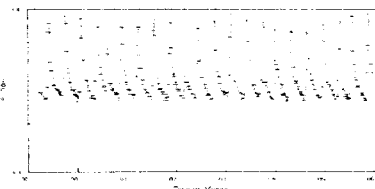
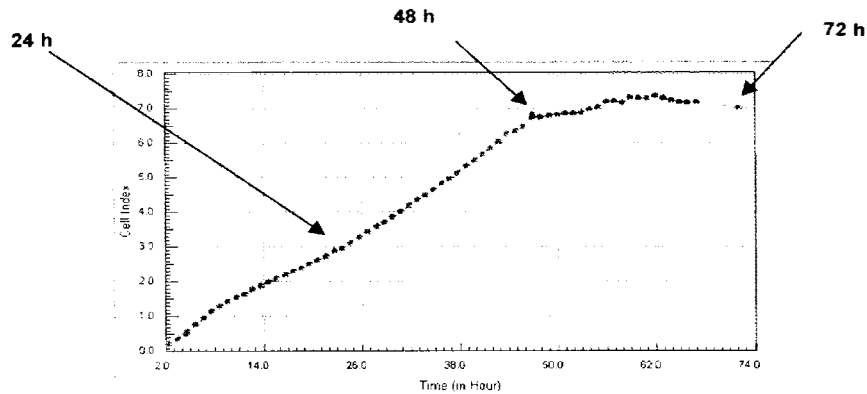
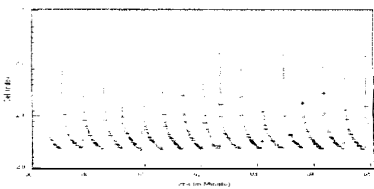
9 / 42

FIG. 6

10 / 42

FIG. 7

Detection of cardiomyocyte beating using the ACEA RT-CES system at different time periods along the growth and differentiative curve of ES-derived cardiomyocytes seeded in ACEA E-Plates. The time resolution between adjacent points in Figures 7A, 7B and 7C is about 40 milliseconds. In other words, a second shown in Figures 7A, 7B and 7C is equivalent to 40 milliseconds.

7A**7B****7C****7D**

11 / 42

FIG. 8A

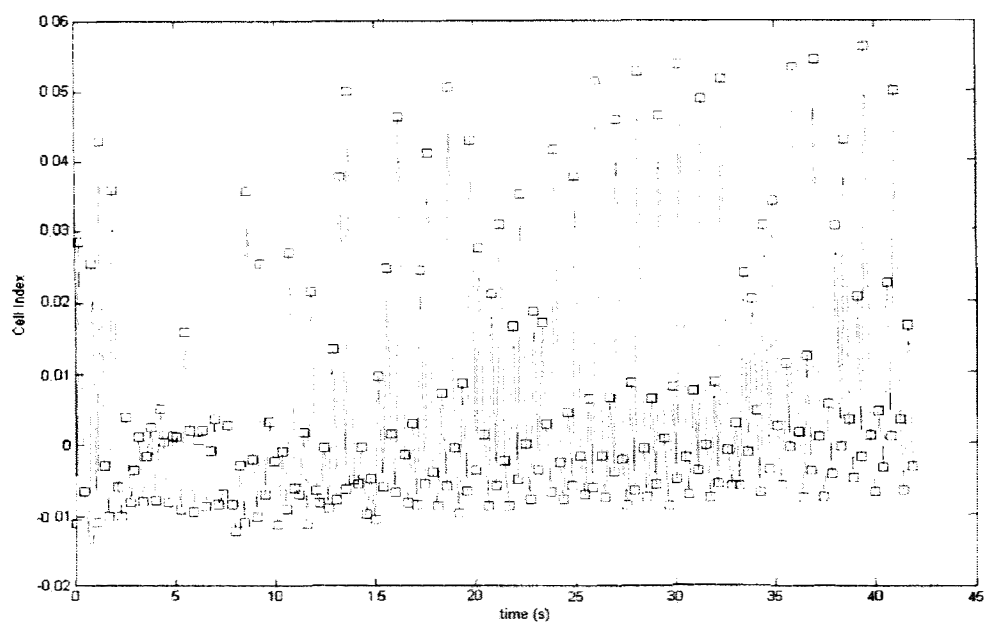
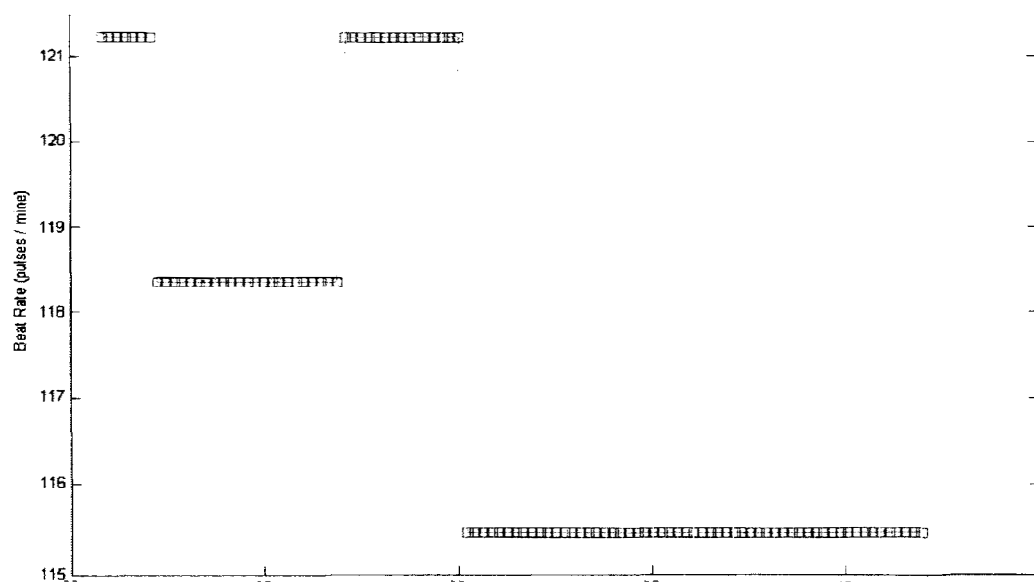
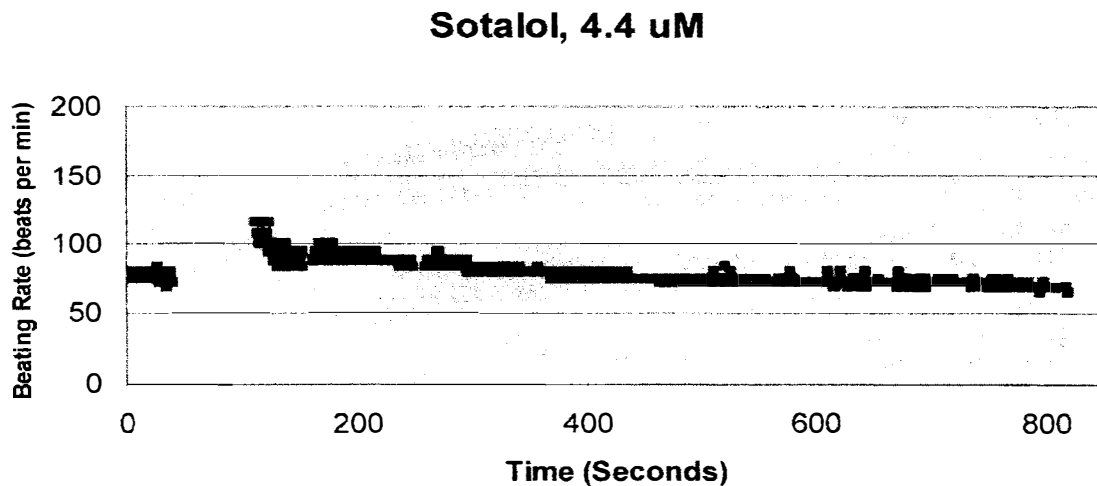
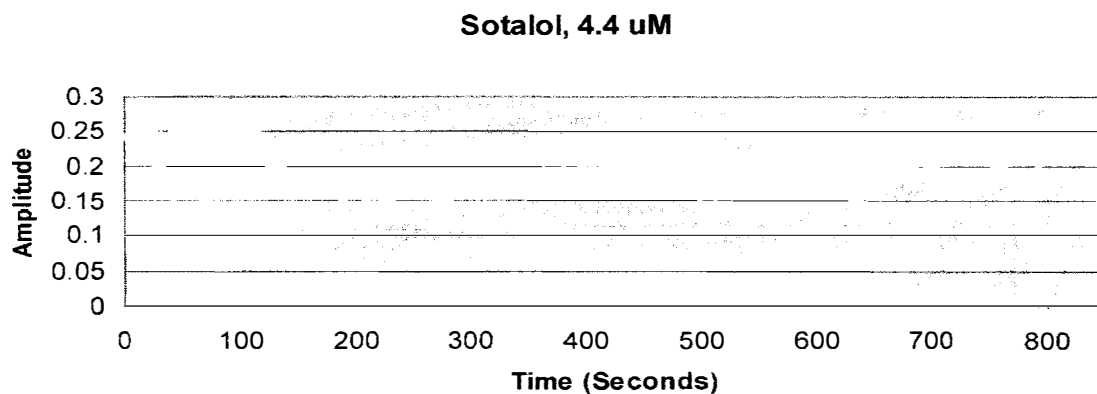


FIG. 8B



12 / 42

FIG. 9 Detection of cardiomyocyte beating using the ACEA RT-CES system for the mouse cardiomyocytes treated with 4.4 μM sotalol. In Figures 9C and 9D, the time resolution between two adjacent points is 40 milliseconds. In other words, a second in Figures 9C and 9D is equivalent to 40-millisecond.

FIG. 9A**FIG. 9B**

13 / 42

FIG. 9C

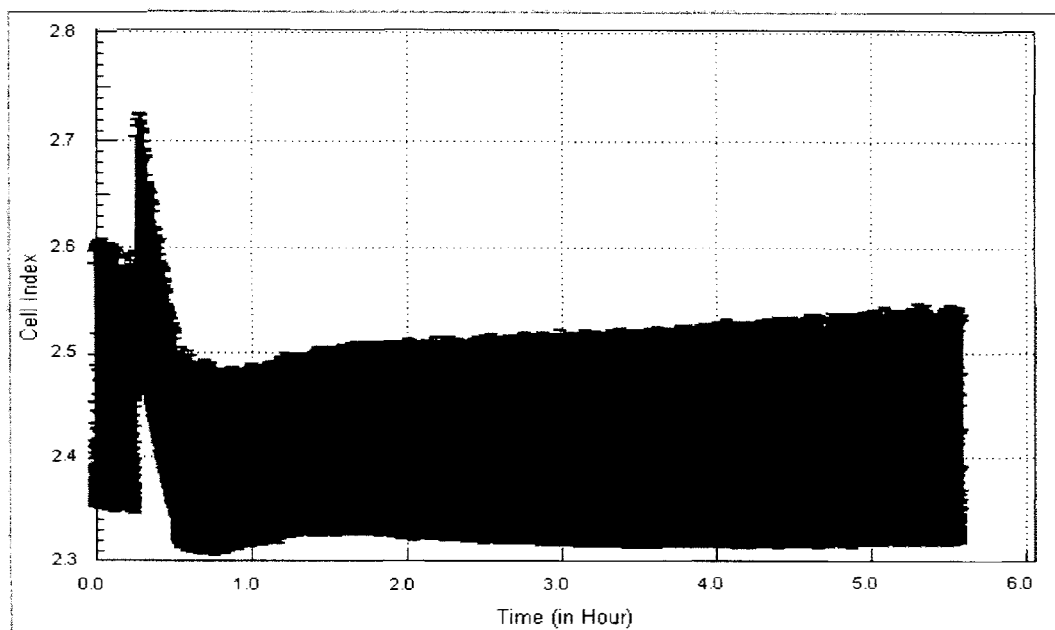
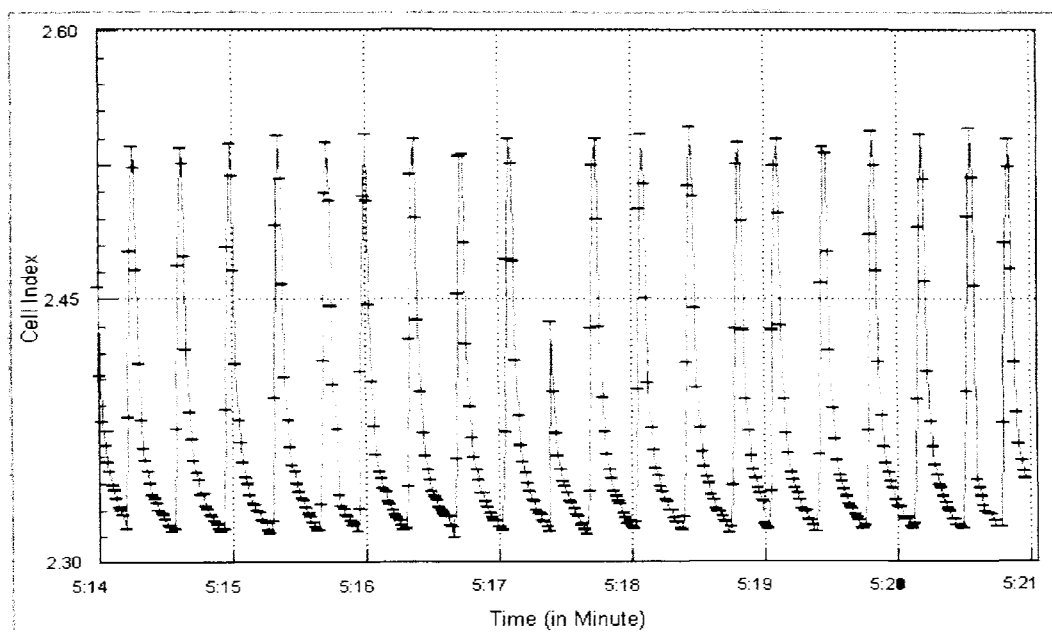
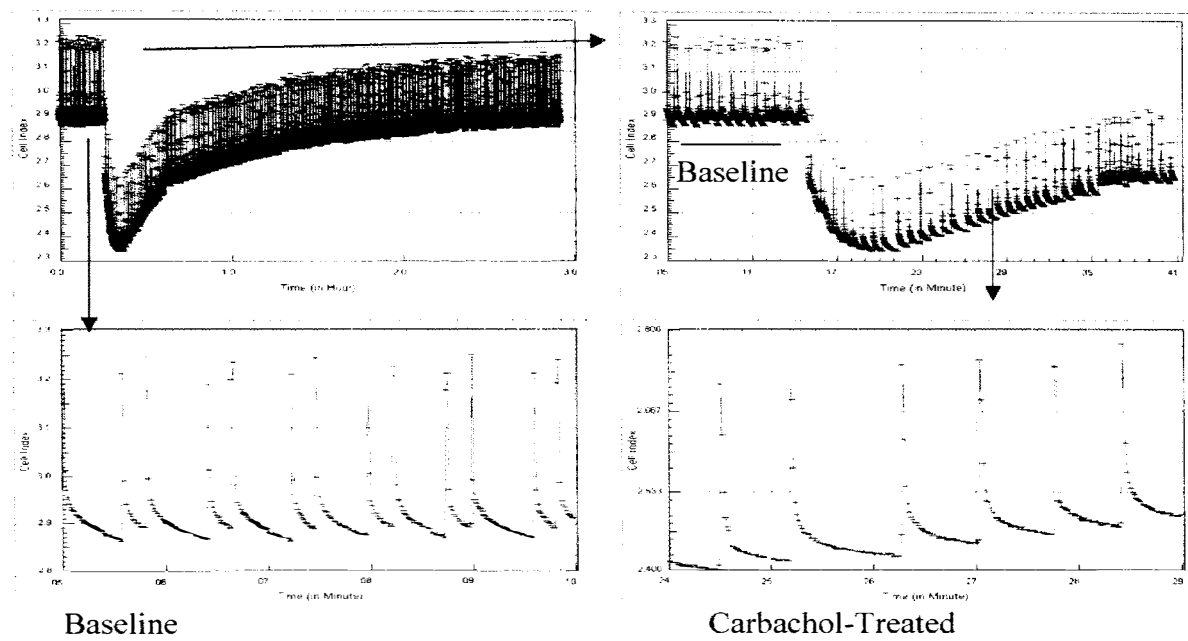


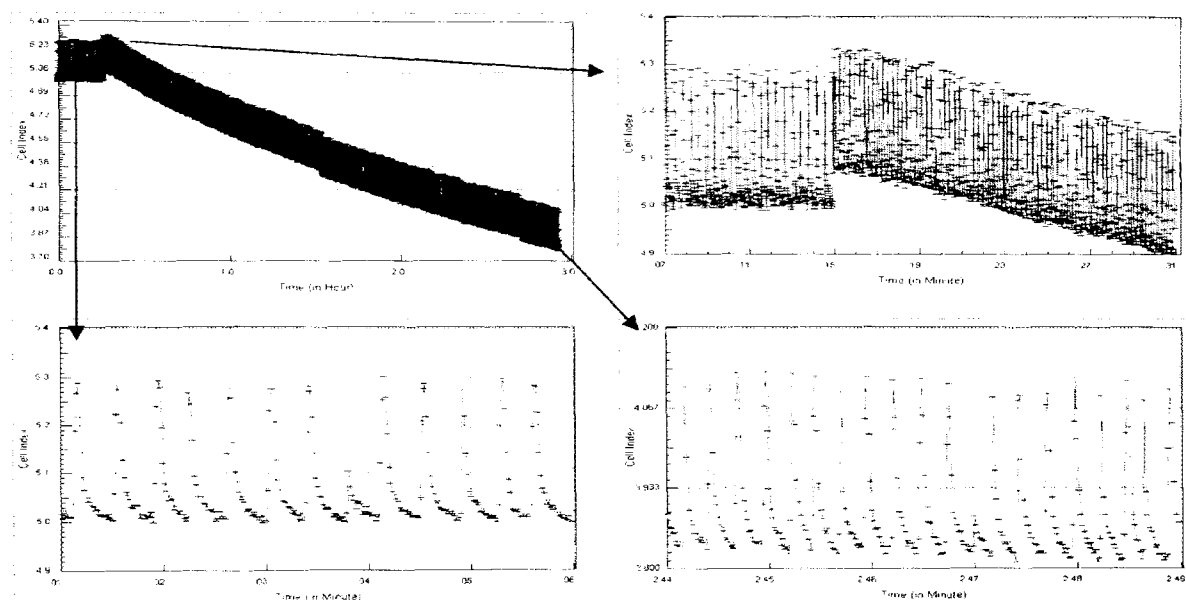
FIG. 9D



14 / 42

FIG. 10A**Assessment of agents which slow down heart rate and cardiomyocyte beating**

15 / 42

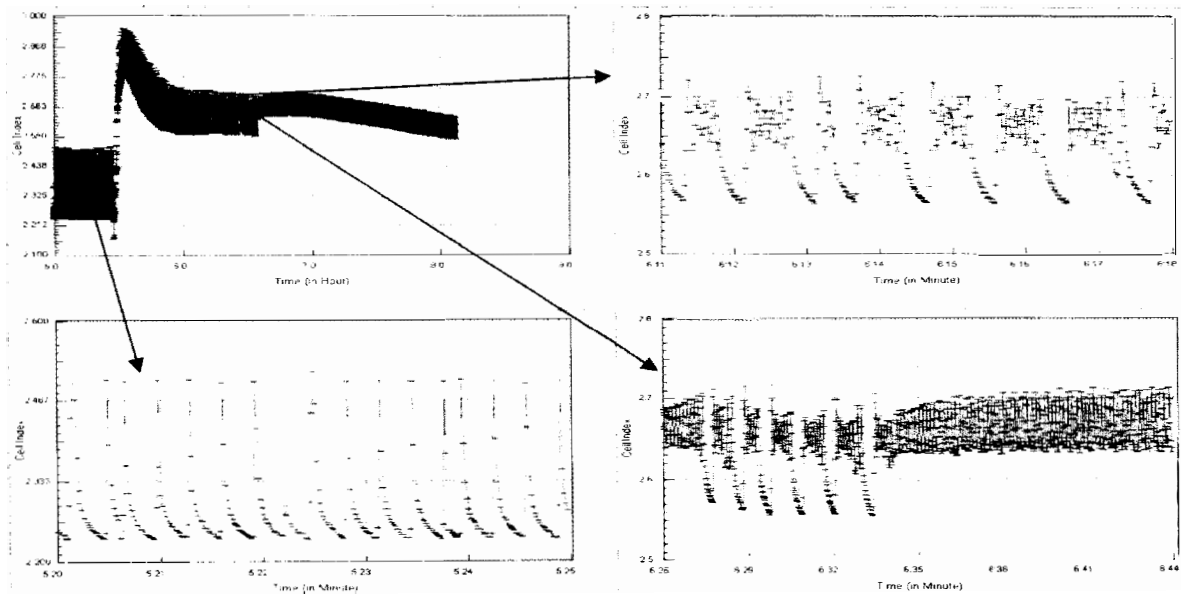
FIG. 10B**Analysis of drugs which increase heart rate and cardiomyocyte beating**

Baseline

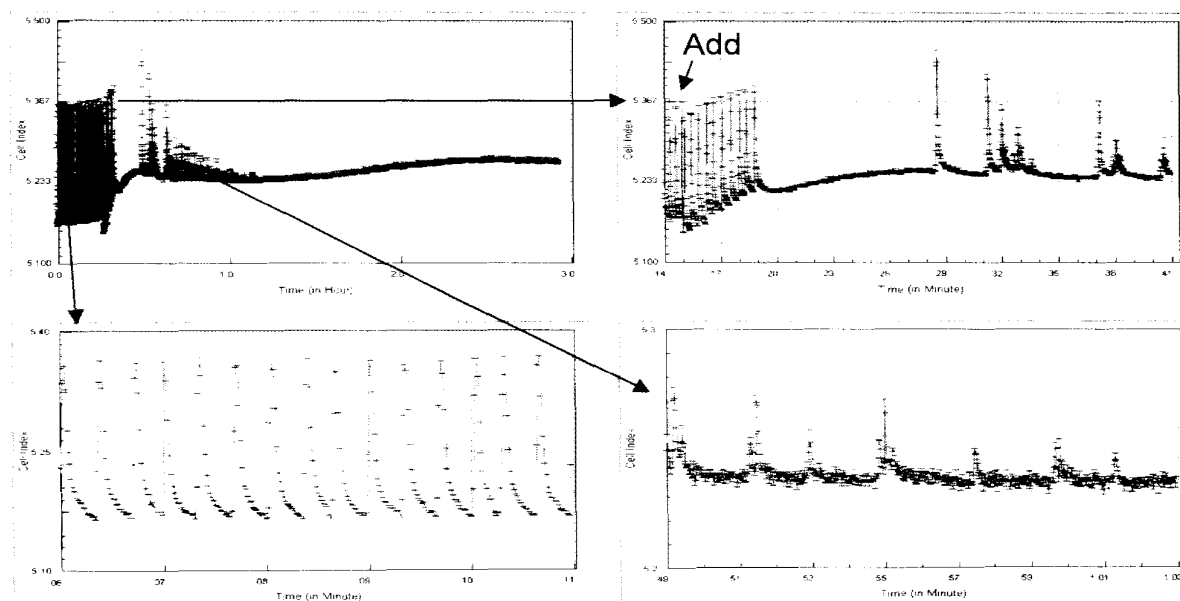
Isoproteranol-treated

16 / 42

FIG. 11A

Sotalolol 33.3 μ M (0.033% DMSO)

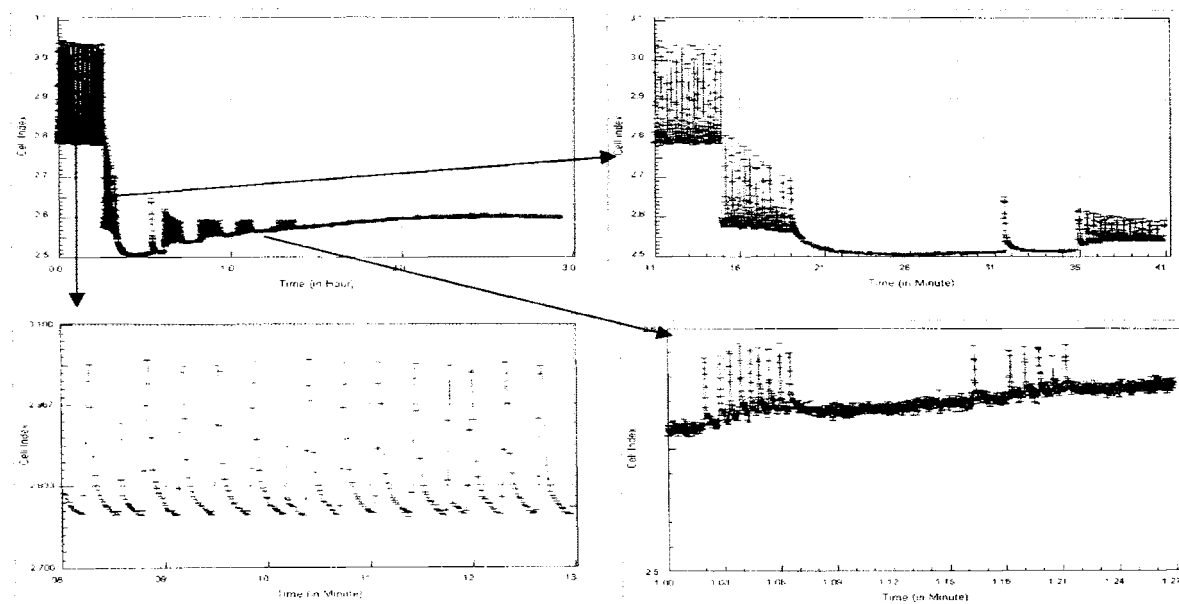
17 / 42

FIG. 11B**Astemizole 400 nM (0.004% DMSO)**

18 / 42

FIG. 11C

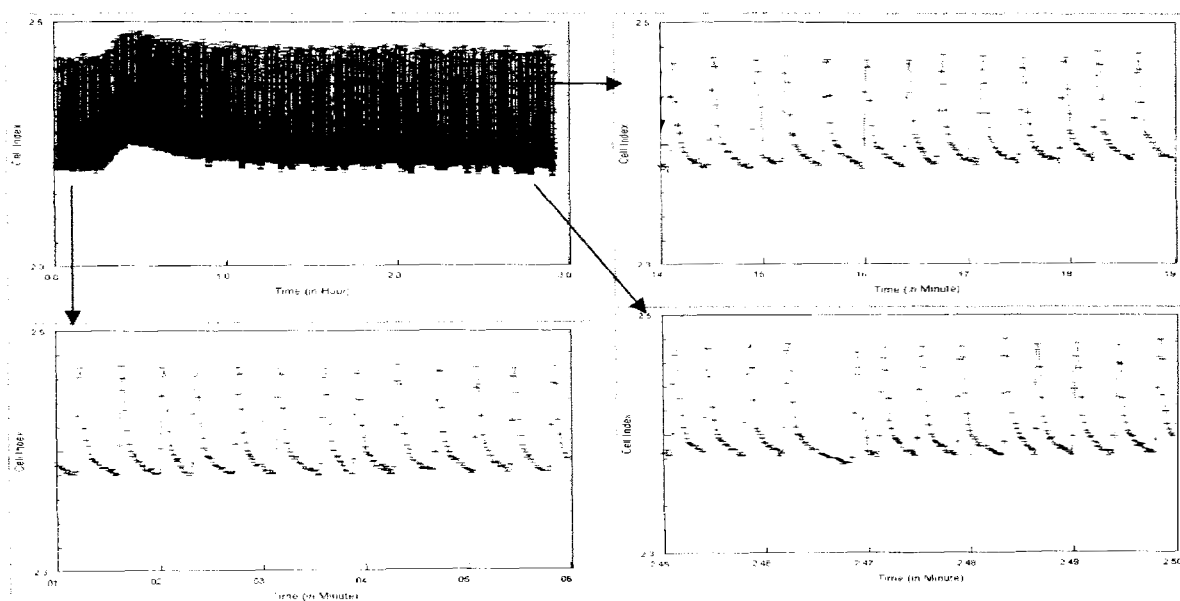
Terfenadine 200 nM (0.065%DMSO)



19 / 42

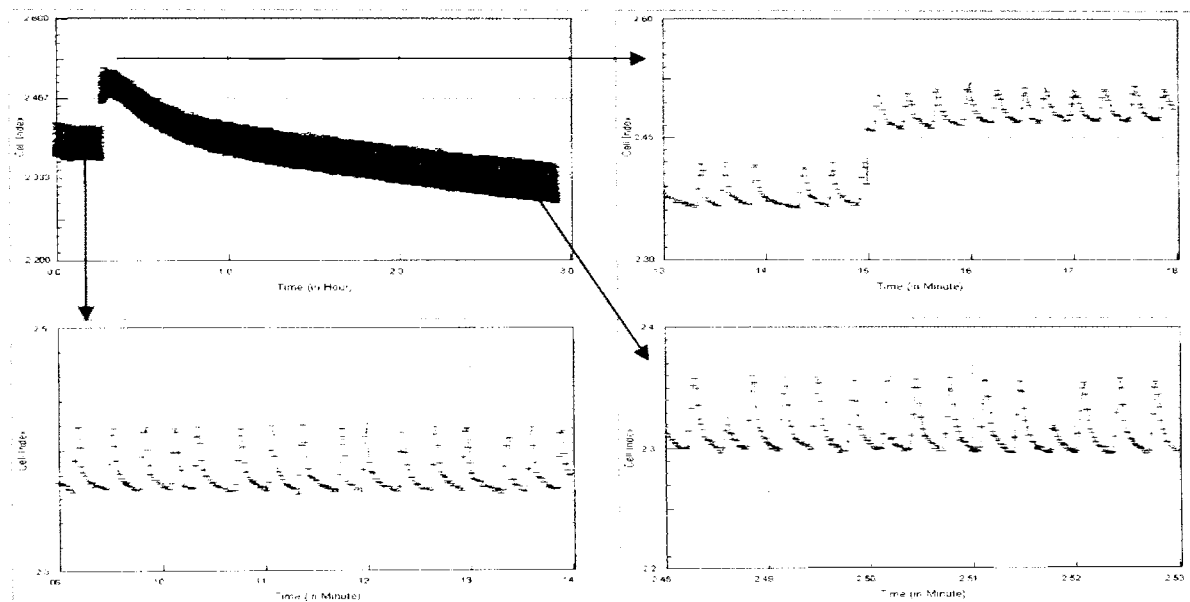
FIG. 11D

Erythromycin 13.3 uM (0.13% DMSO)



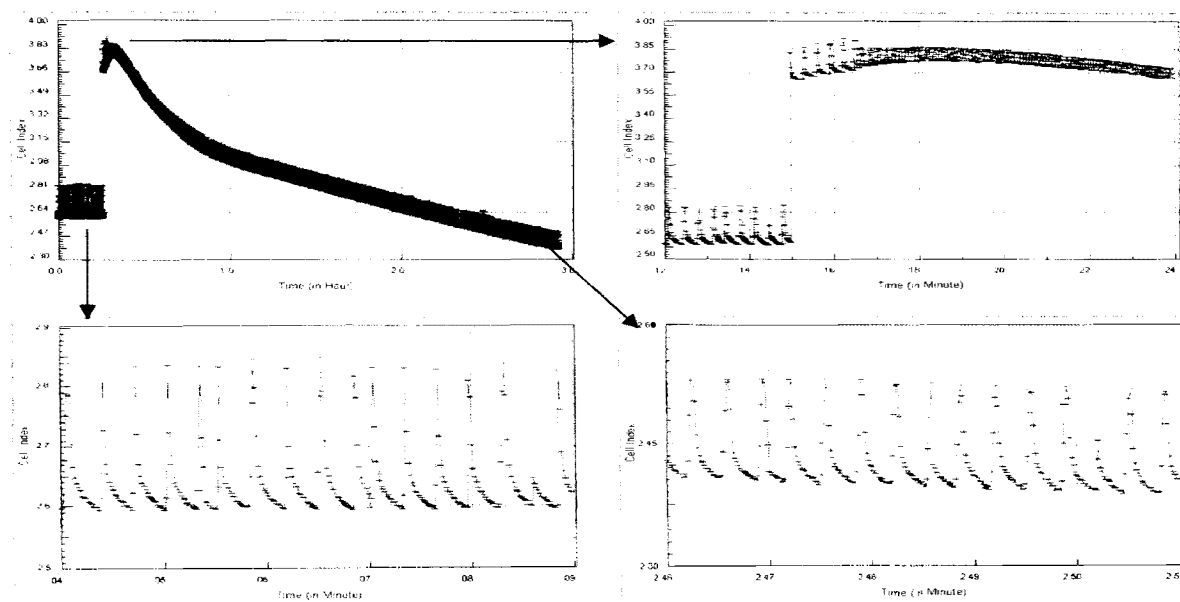
20 / 42

FIG. 11E

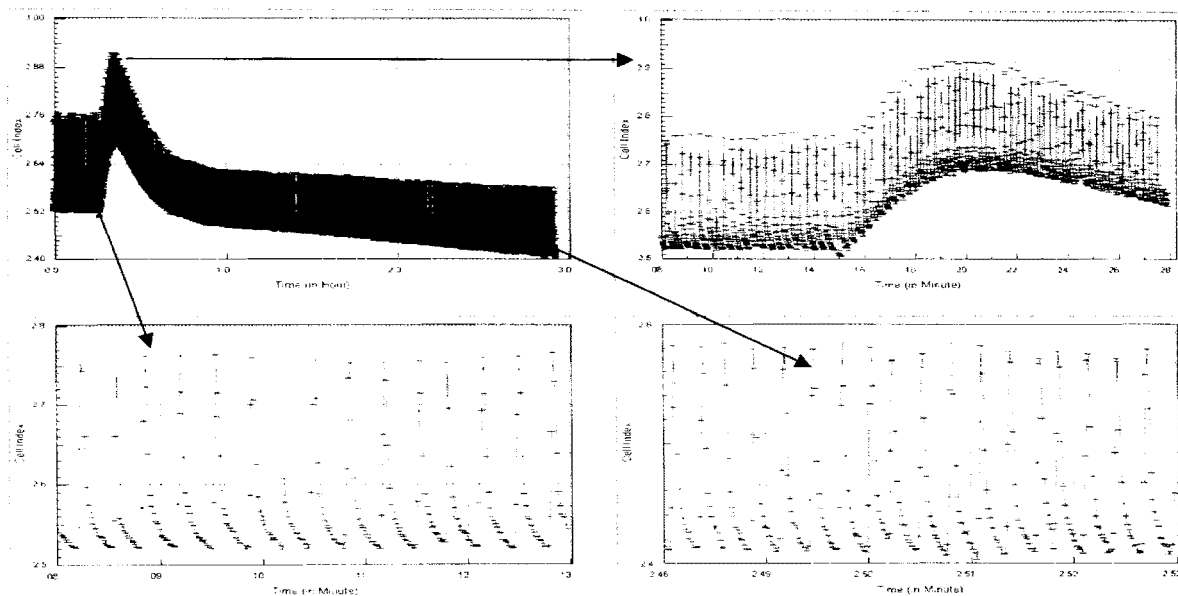
Moxifloxacin 20 μ M (0.3% DMSO)

21 / 42

FIG. 11F

Pentamidine 20uM (0.2%DMSO)

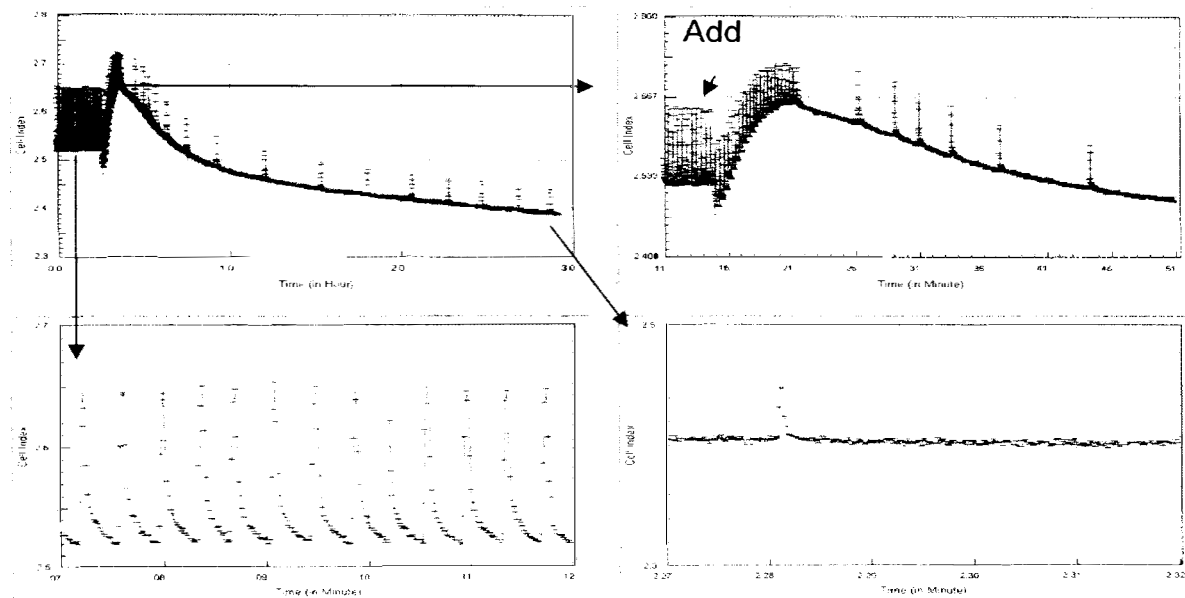
22 / 42

FIG. 11G**Amitriptyline 4.4uM(0.04 % DMSO)**

23 / 42

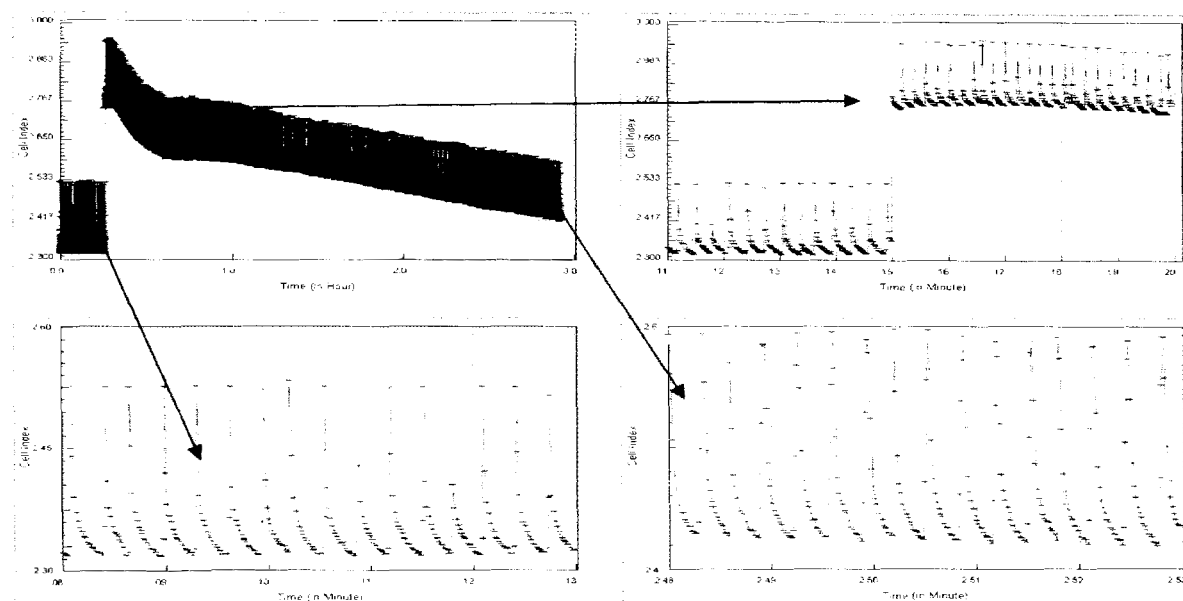
FIG. 11H

Verapamil 130 nM (0.0013% DMSO)



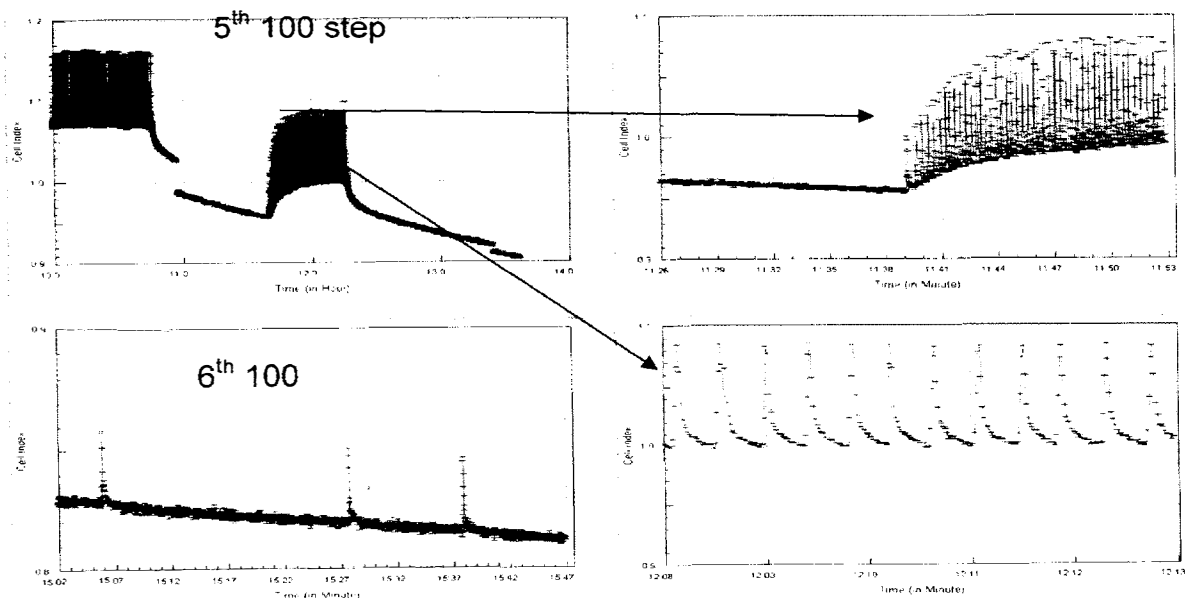
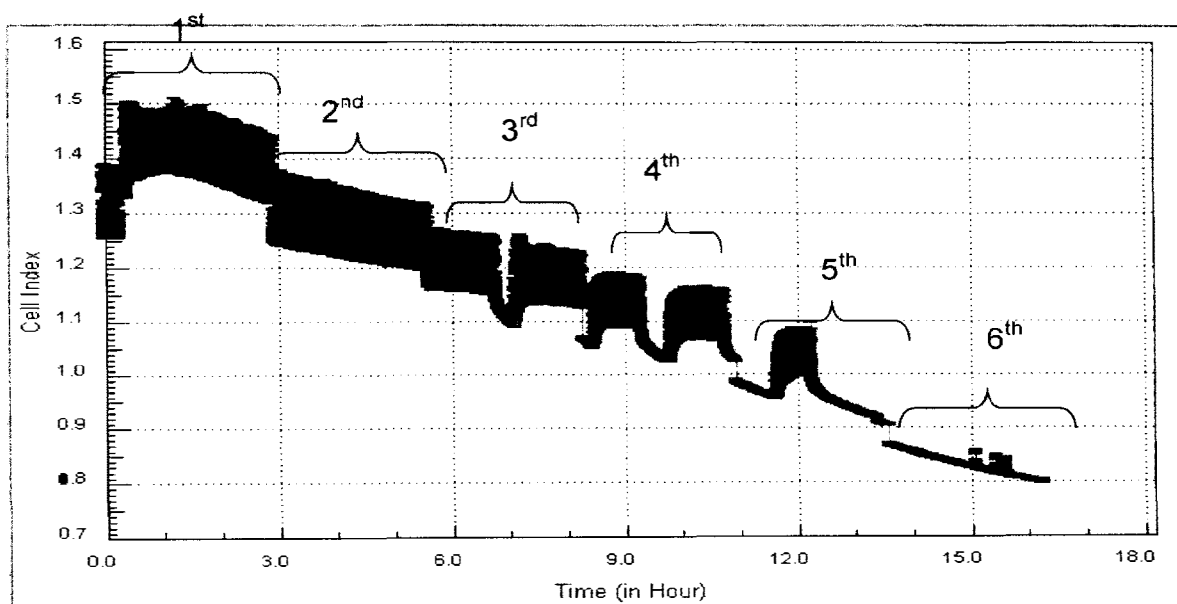
24 / 42

FIG. 11I

Rosiglitazone 13.3 μ M (0.13% DMSO)

25 / 42

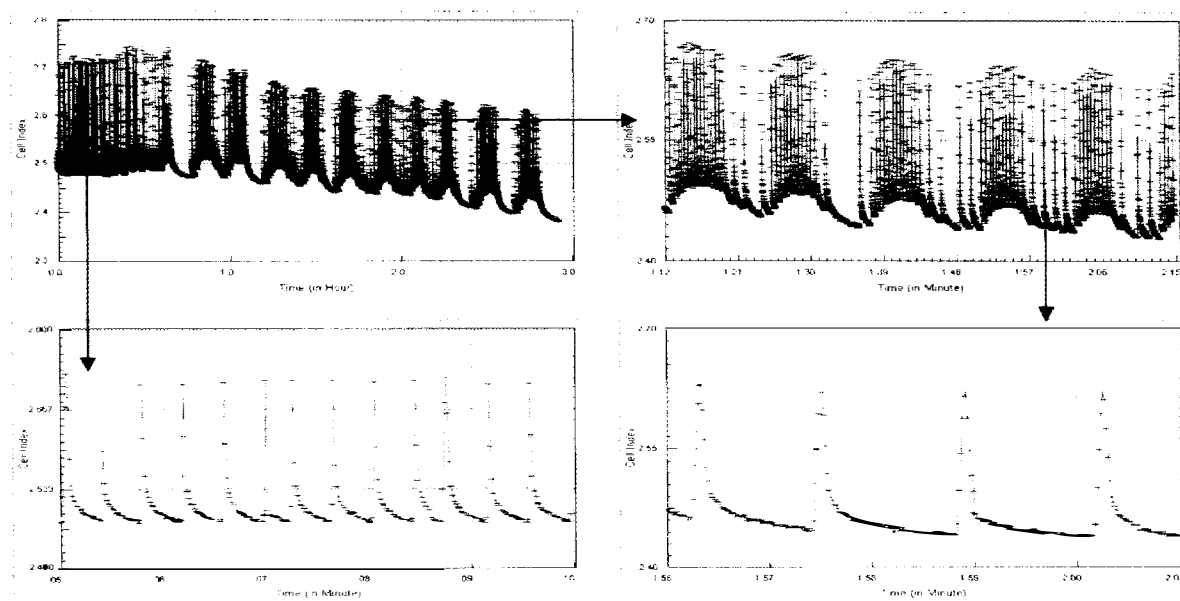
FIG. 11J

Rofecoxib 13.3 μ M

26 / 42

FIG. 11K

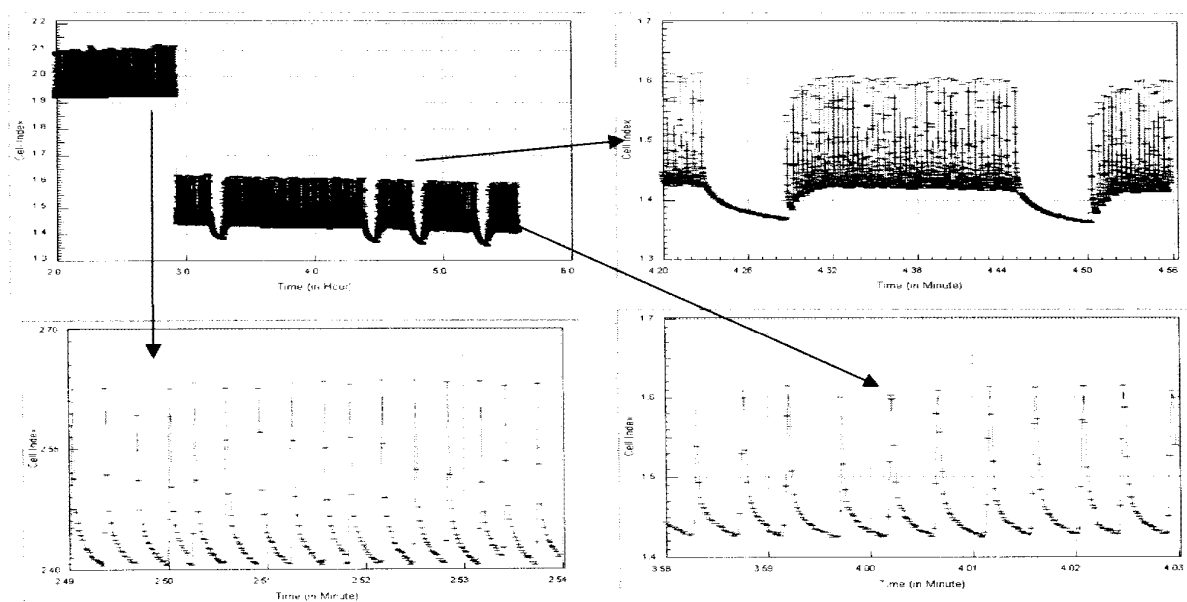
Celecoxib 4.4 uM



27 / 42

FIG. 11L

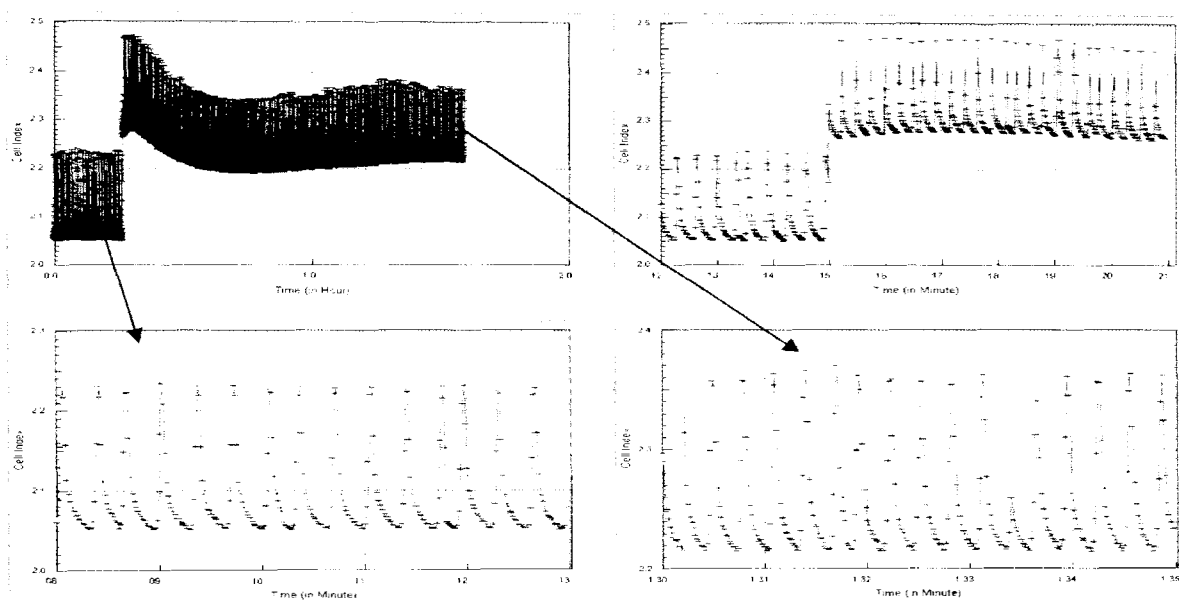
Doxirubicin 40 uM (0.4% DMSO)
(measurement after 2h treatment)



28 / 42

FIG. 11M

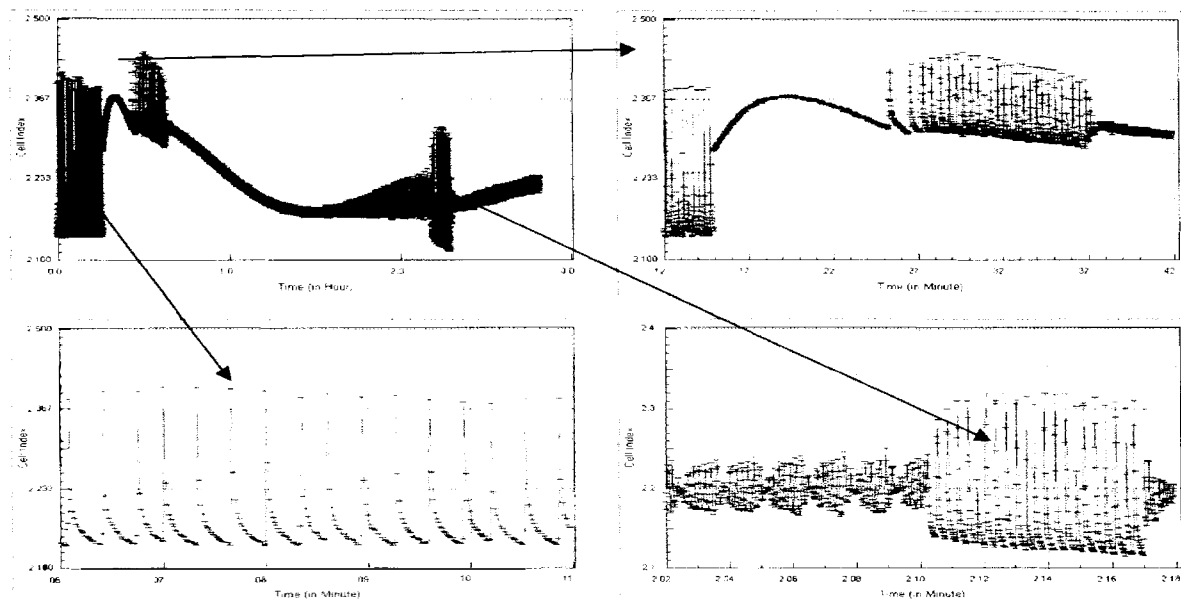
Cyclosporine A 13.3uM (0.13% DMSO)



29 / 42

FIG. 11N

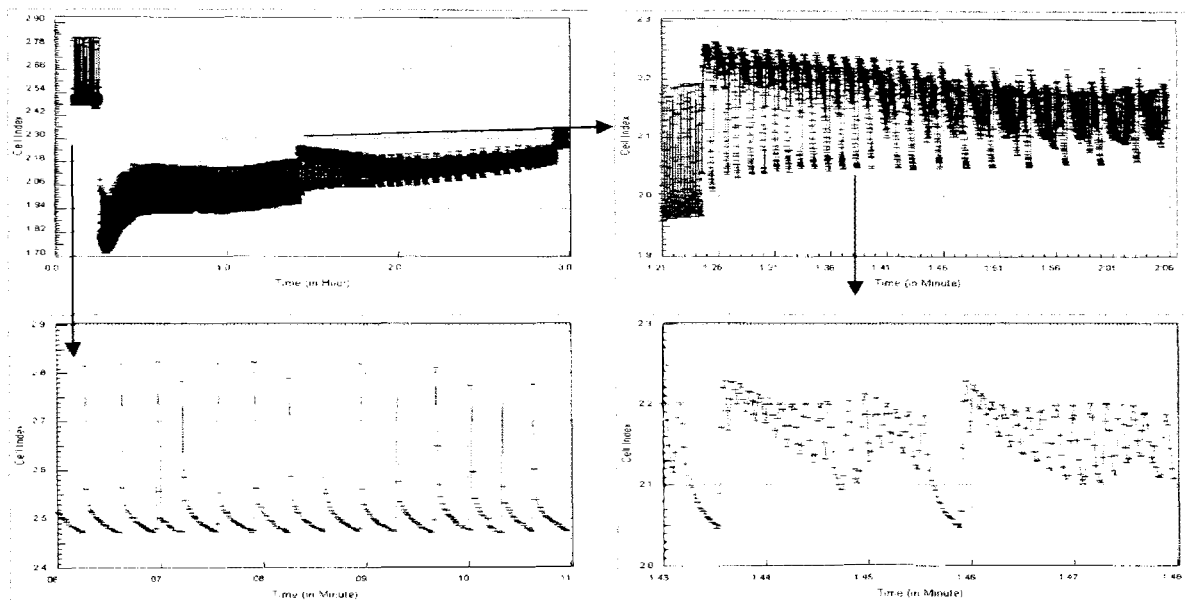
Propranolol 4.4uM (0.04% DMSO)



30 / 42

FIG. 110

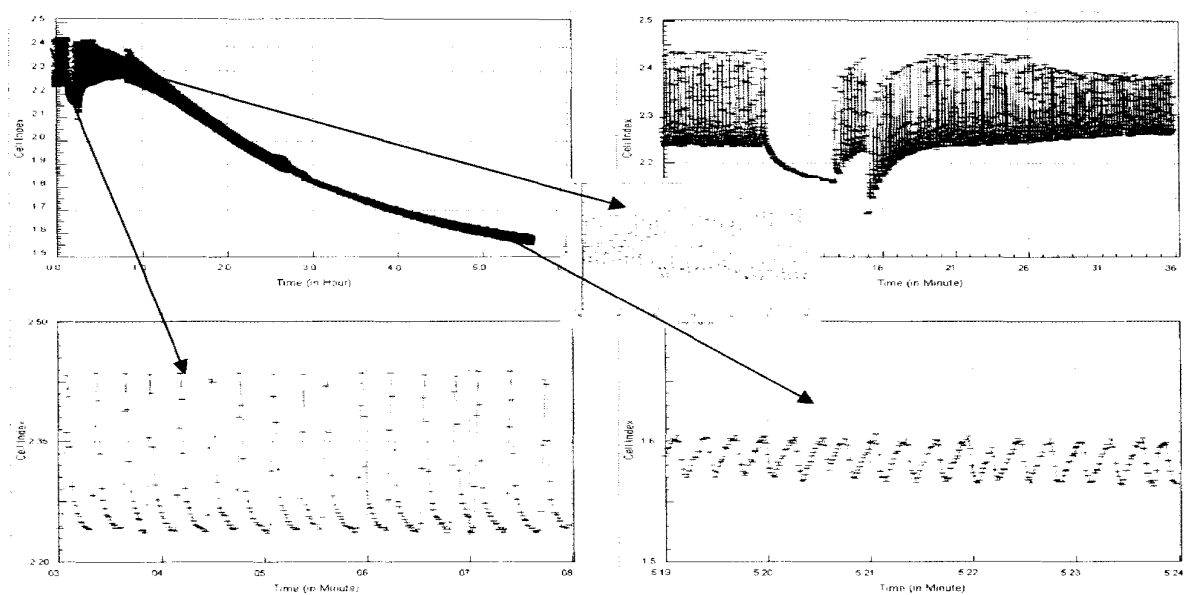
E4031 9.1 nM



31 / 42

FIG. 11P

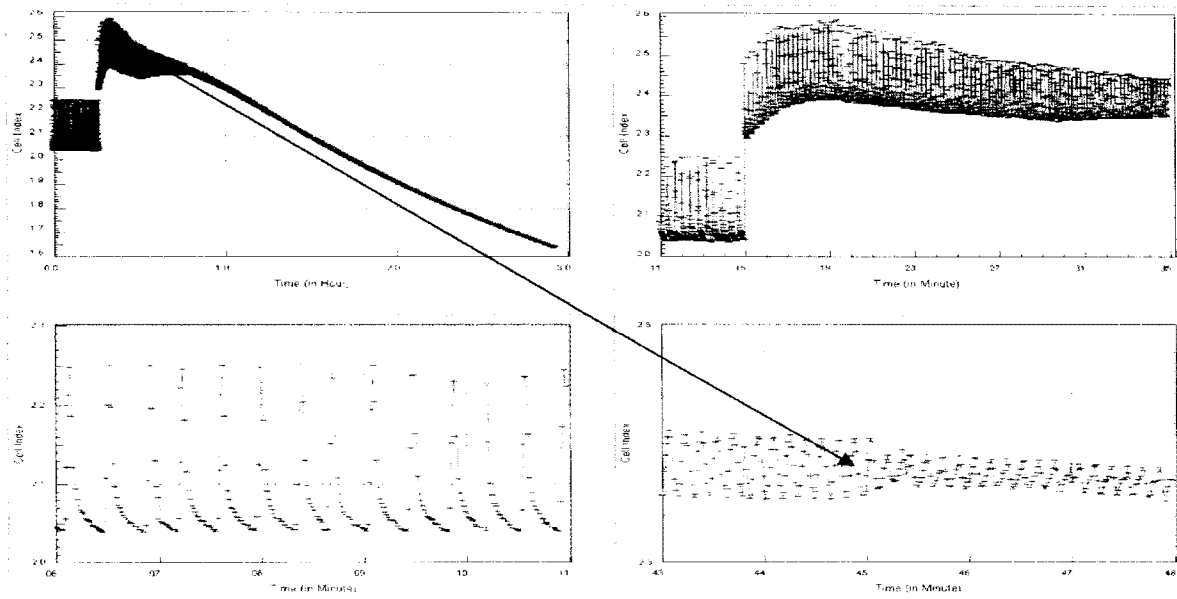
DDT 8uM (0.08% DMSO)



32 / 42

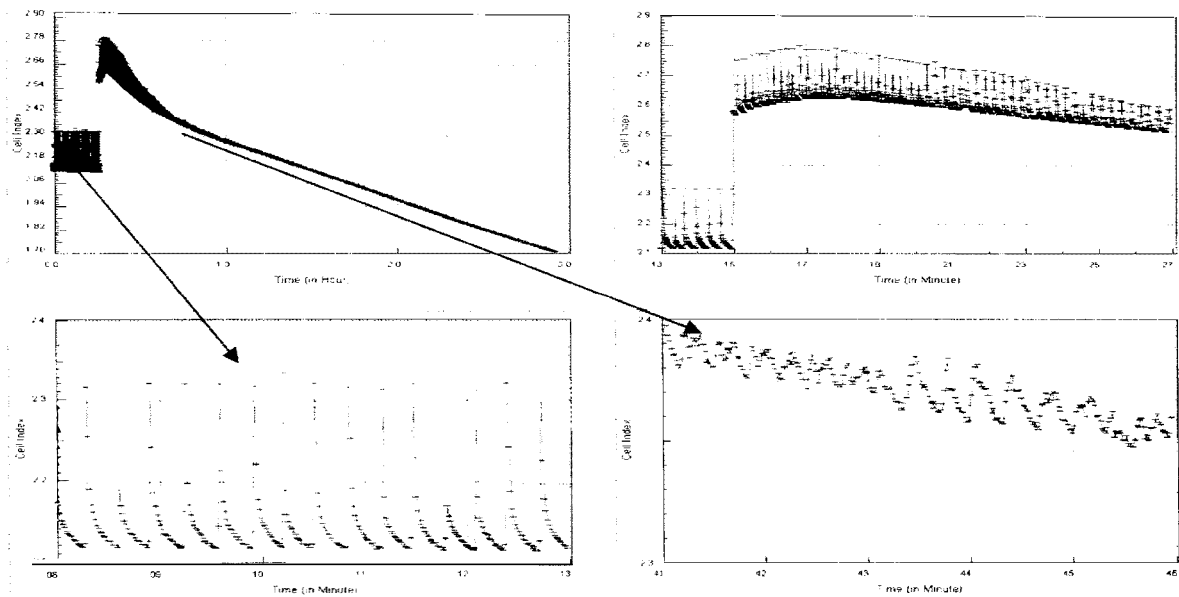
FIG. 11Q

PCB 8uM (DMSO 0.08%)



33 / 42

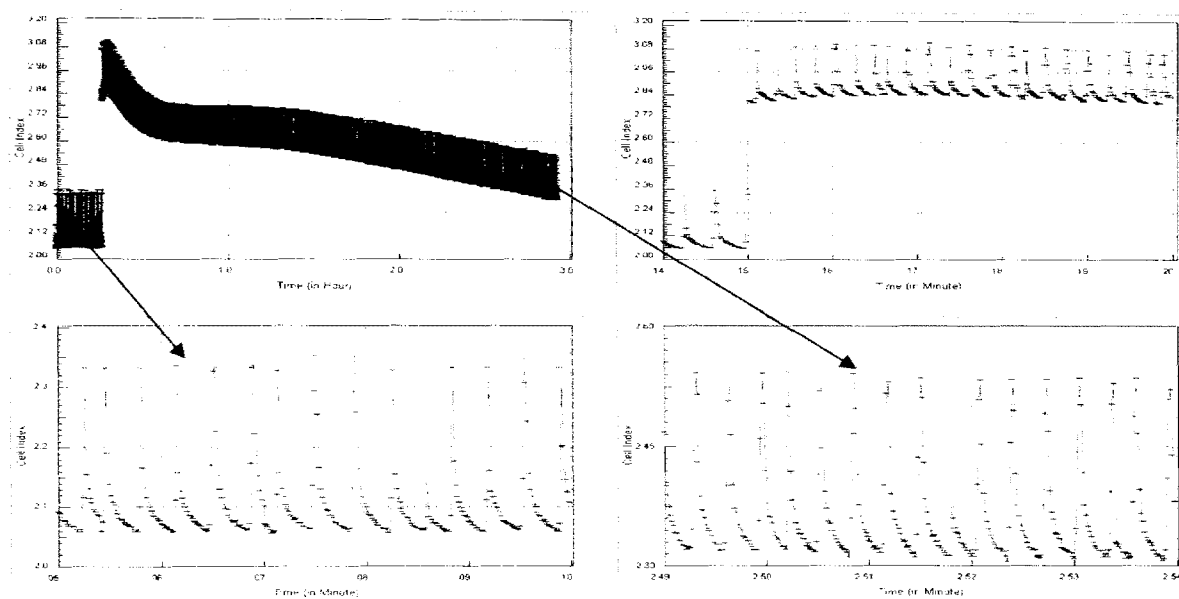
FIG. 11R

Endosulfan 8uM (0.08% DMSO)

34 / 42

FIG. 11S

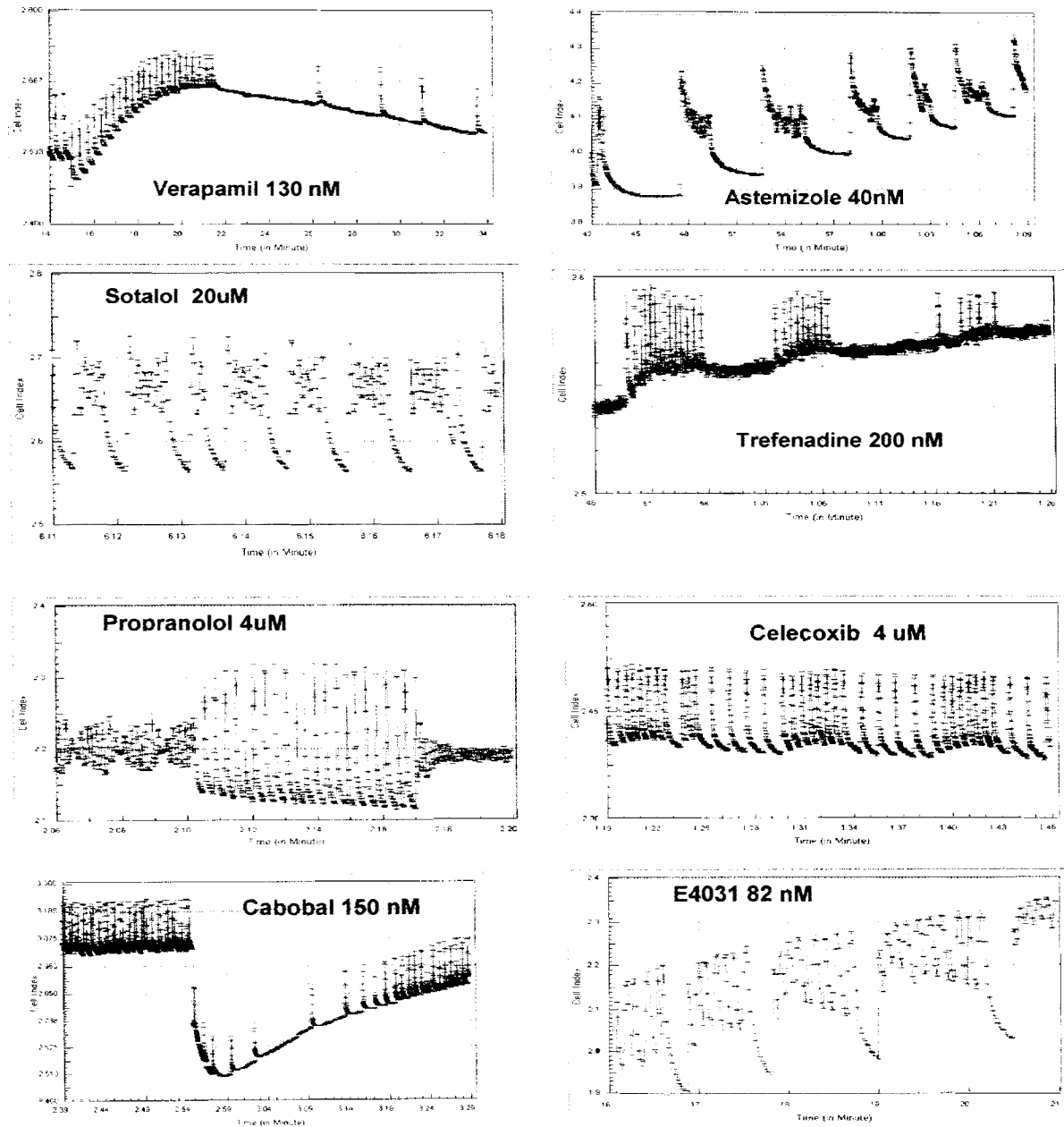
DMSO 0.13%



35 / 42

FIG. 12

Cardiotoxic Patterns



36 / 42

FIG. 13.

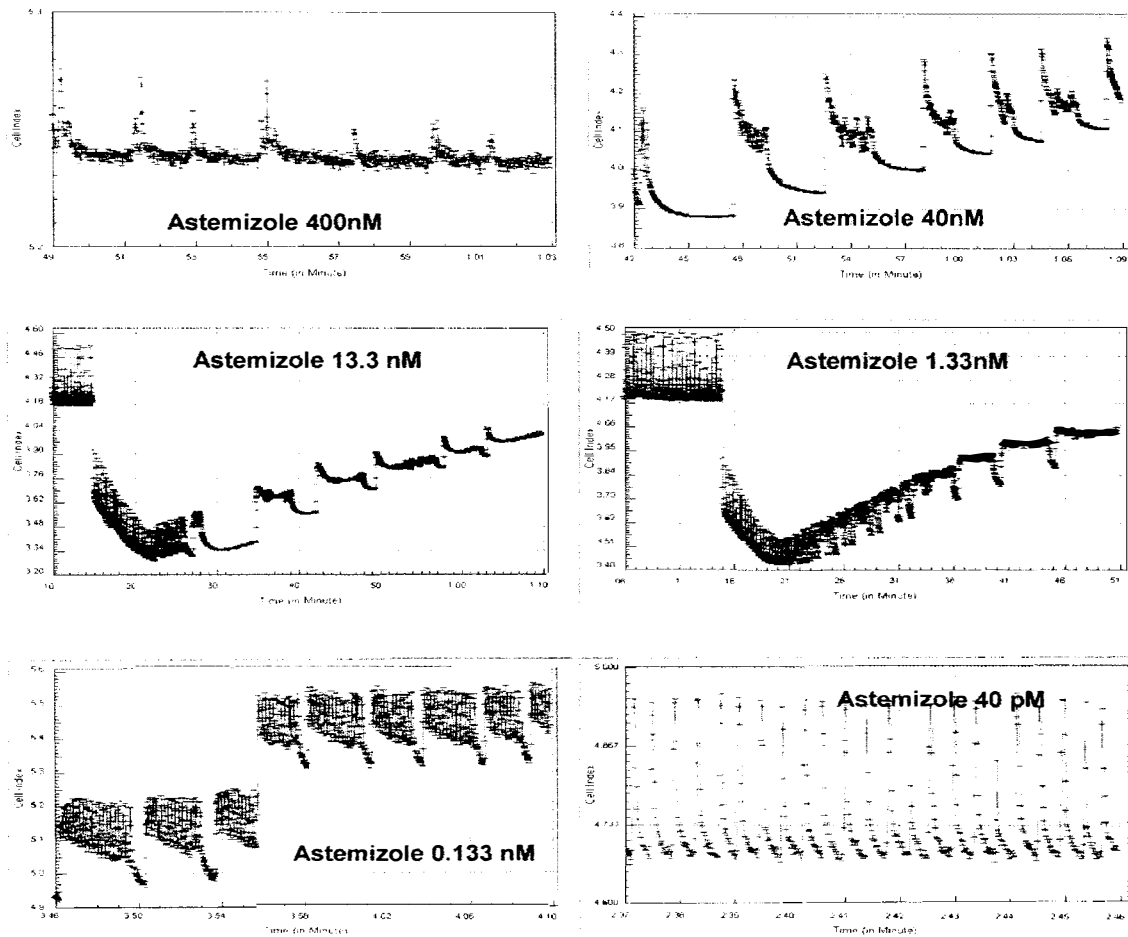


FIG. 14

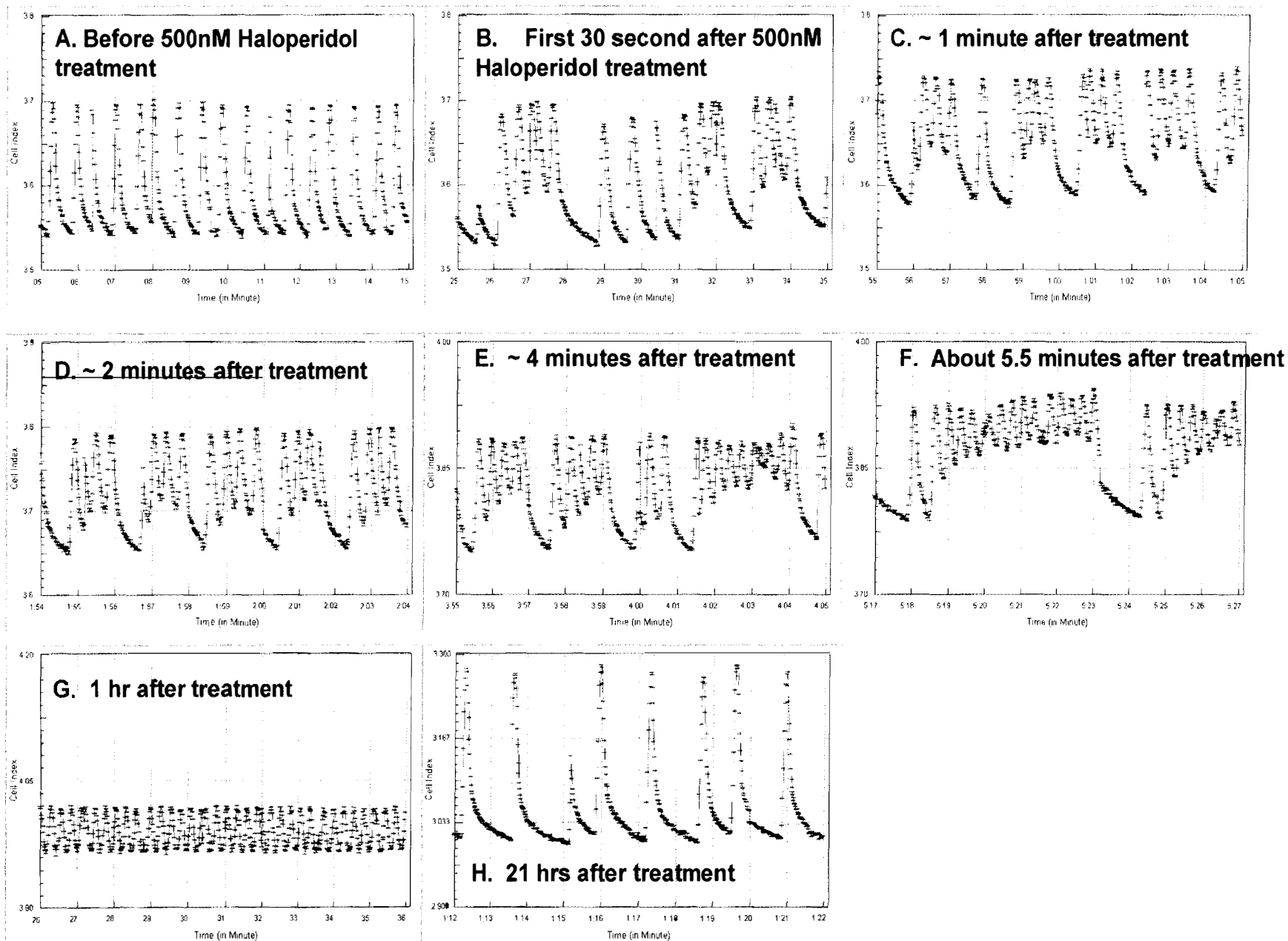


FIG. 15

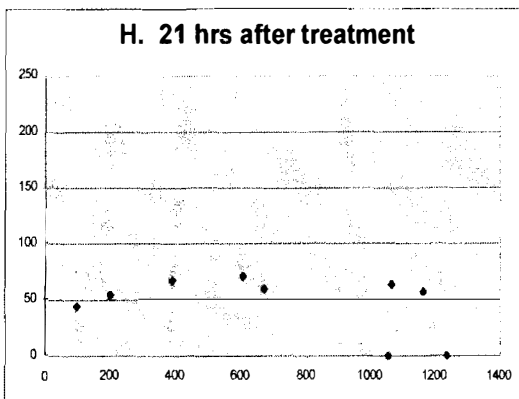
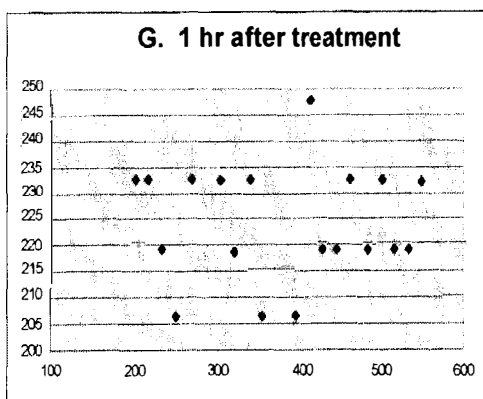
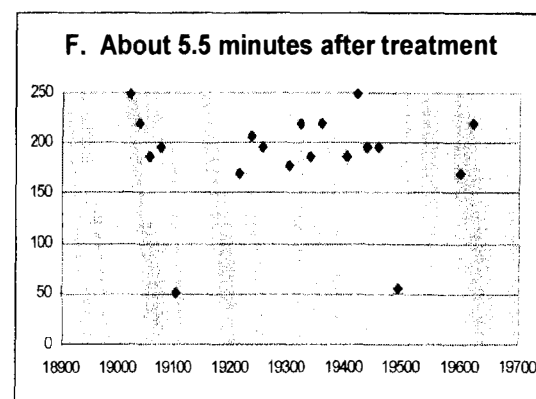
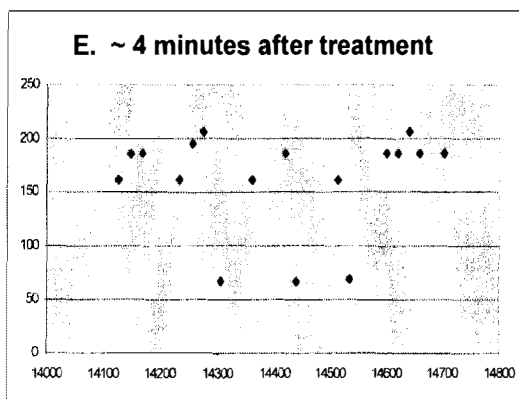
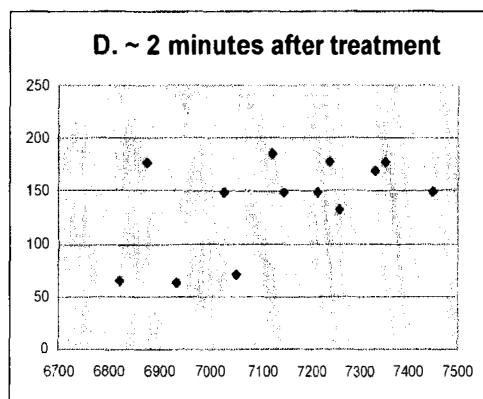
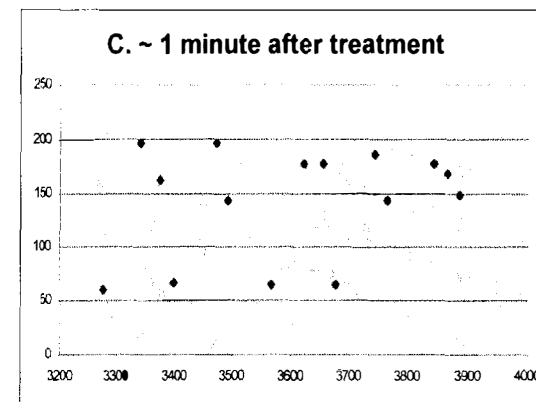
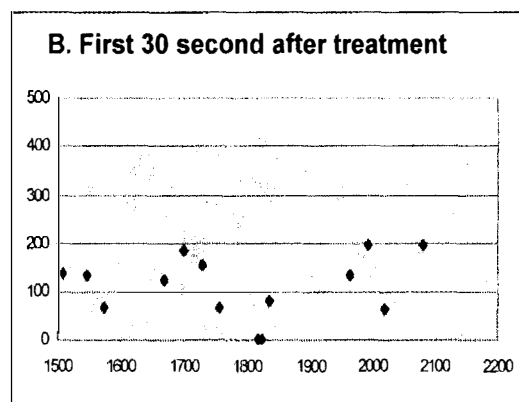
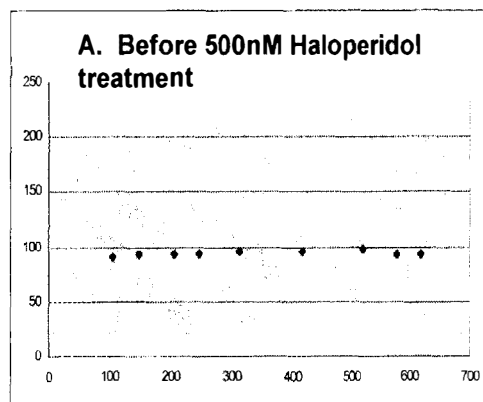


FIG. 16

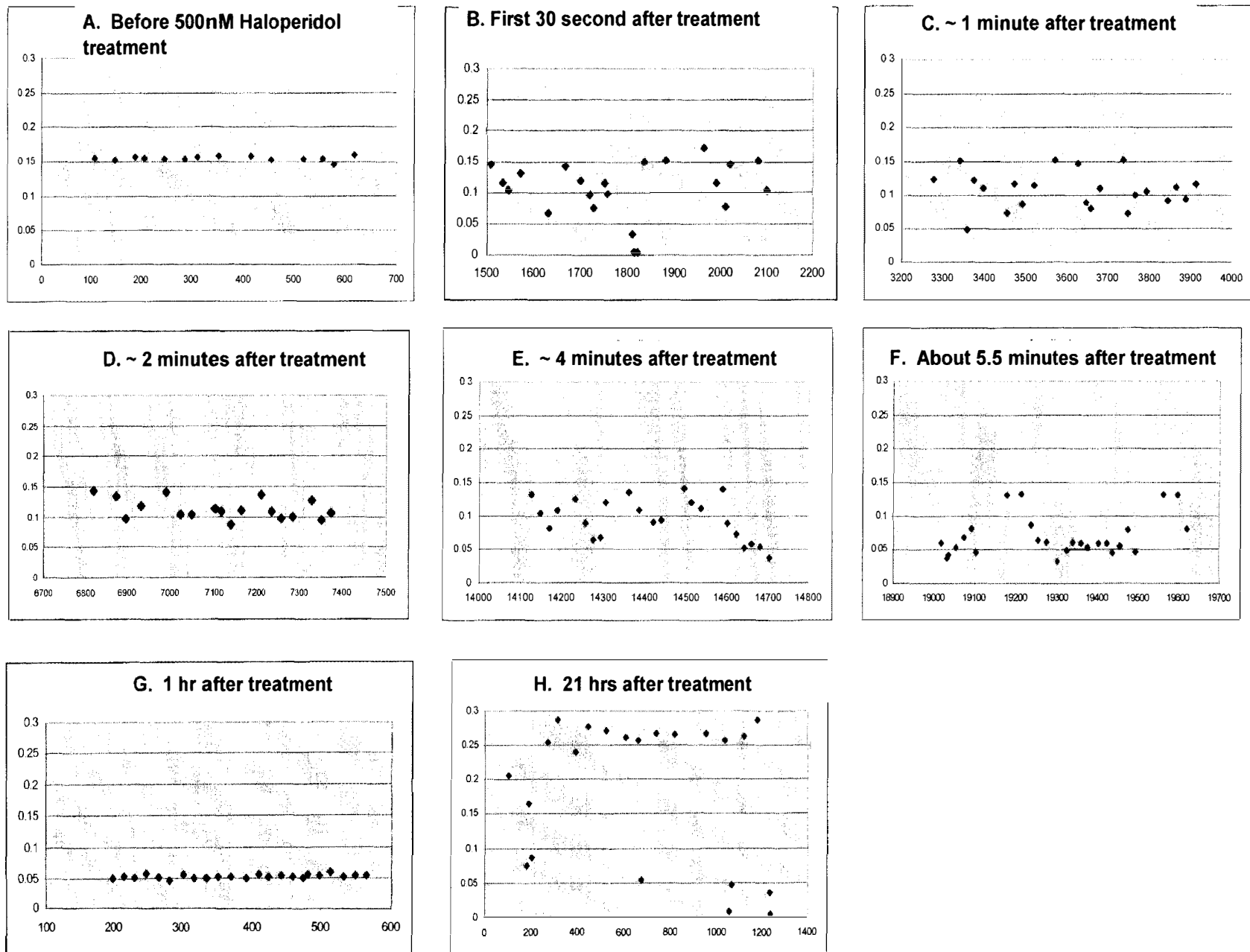


FIG. 17

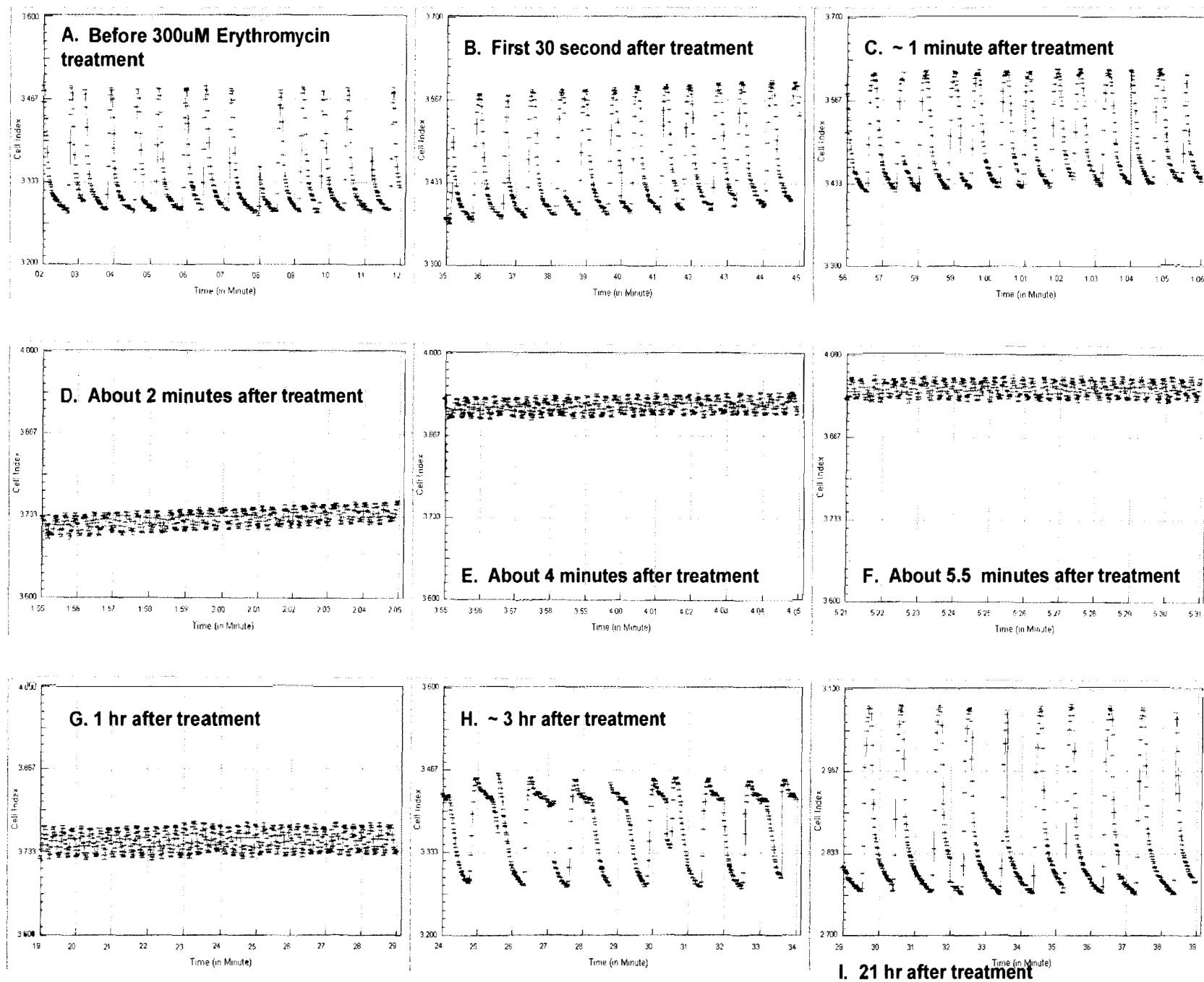


FIG. 18

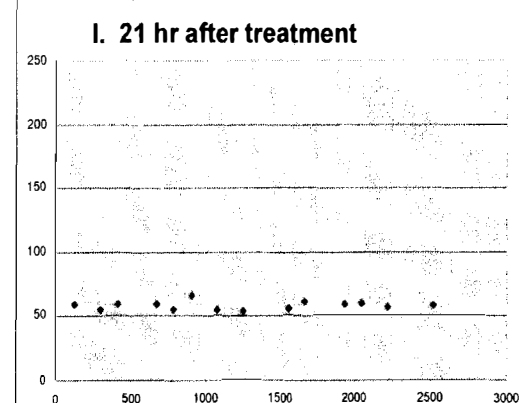
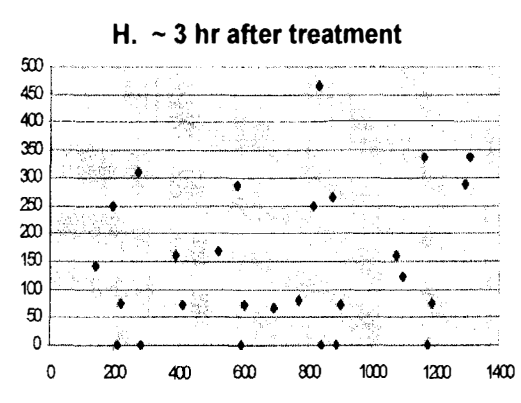
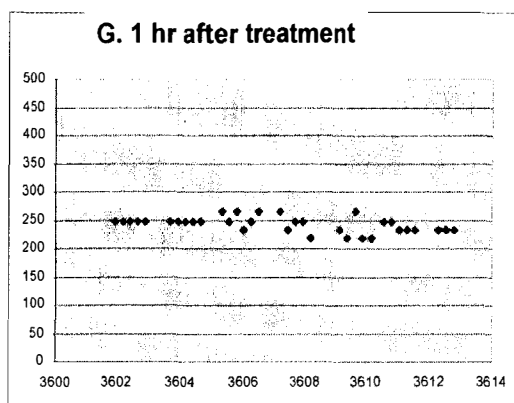
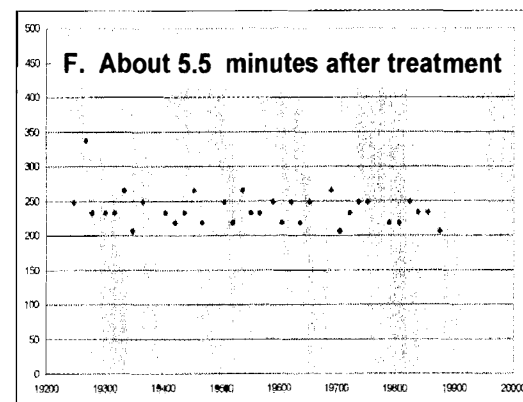
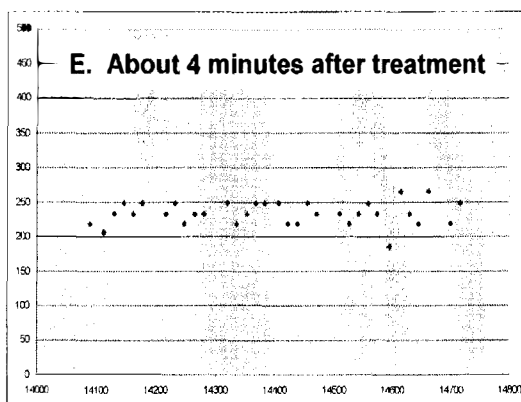
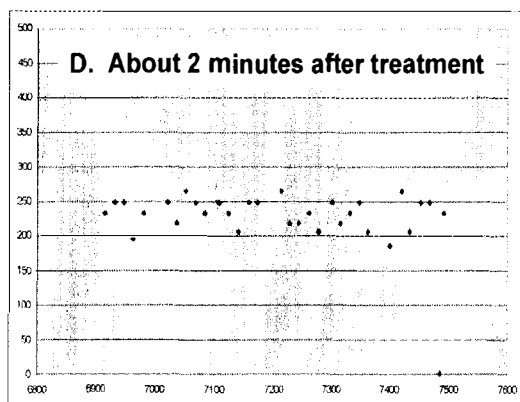
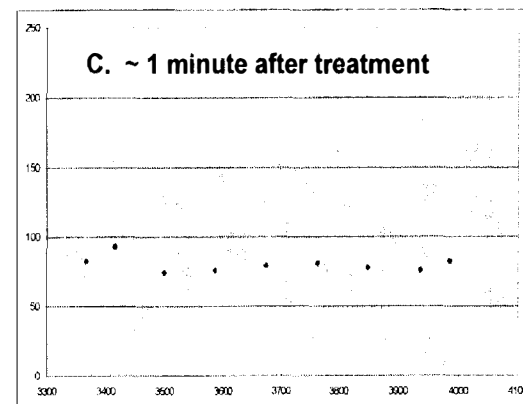
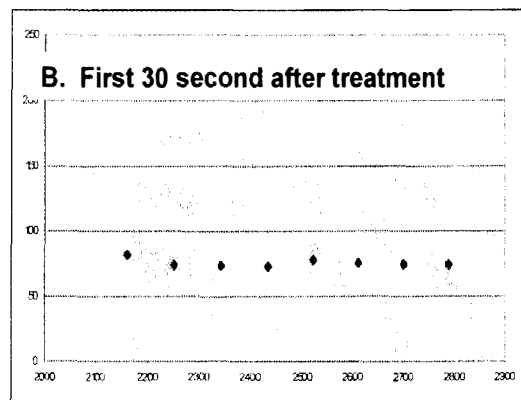
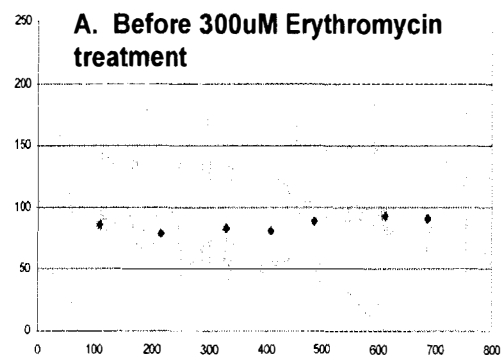
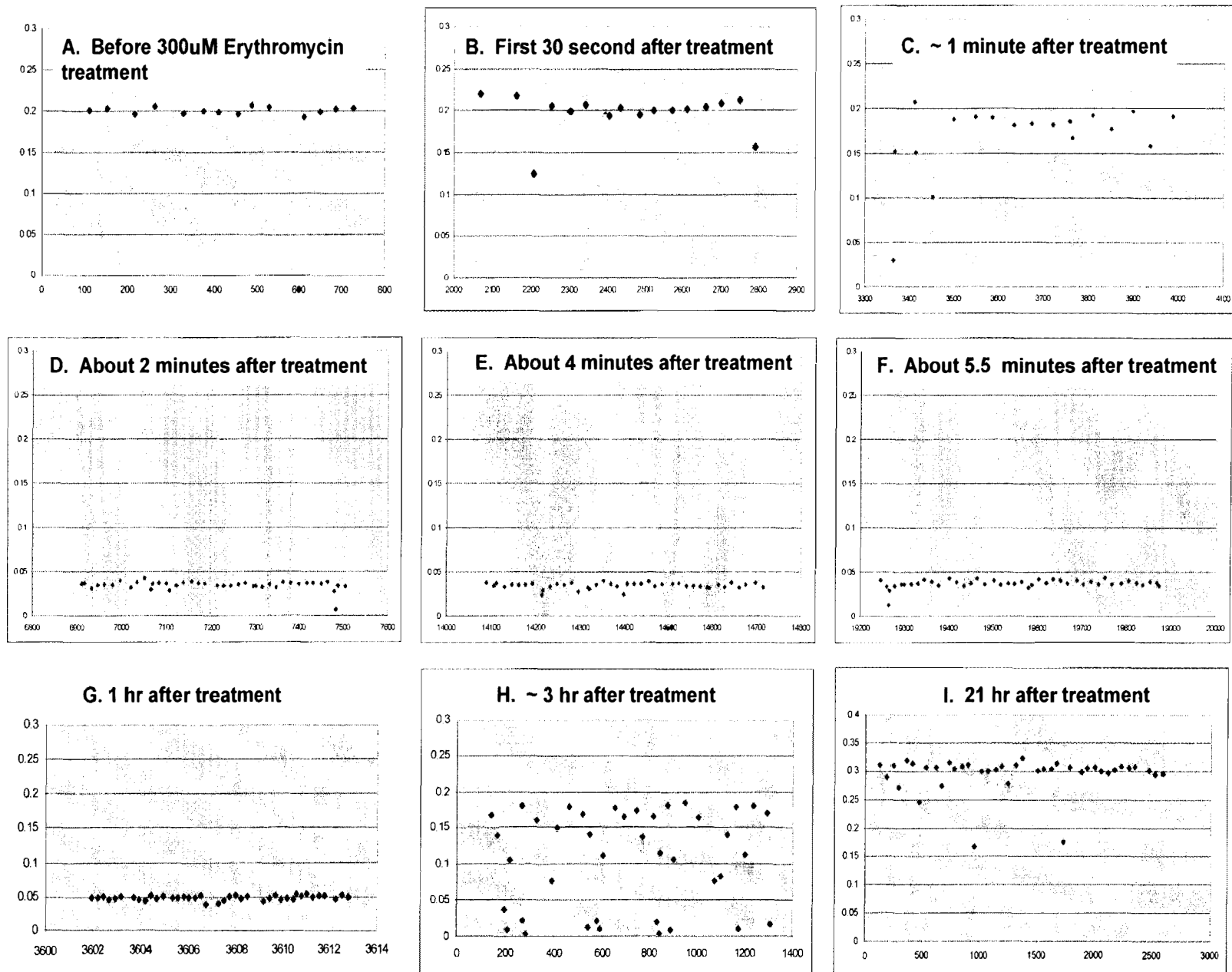


FIG. 19



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/42787

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 27/02; C12M 1/34 (2009.01)

USPC - 435/288.4; 436/149

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8):G01N 27/02; C12M 1/34 (2009.01)

USPC: 435/288.4; 436/149

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 435/283.1, 435/287.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWest, Google Scholar, Google Patent: monitor, cell-substrate, impedance, nanoconductive, electrode array\$2, fabricated, well\$2, surface, attach\$5, analyz\$4, millisecond, intervals, electronic circuitry, record\$2, metal, interdigitated, cardiomyocytes, beating cells, in vitro

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	2007/0172939 A1 (XU et al.) 26 Jul 2007 (26.07.2007); para [0015]-, [0016], [0074], [0098], [0109], [0114], [0120], [0183], [0240], [0274]	1-39
Y	US 2006/0161073 A1 (SINGER et al.) 20 Jul 2006 (20.07.2006); para [0002], [0003], [0018]	1-39
Y	US 2006/0057771 A1 (KOVACS et al.) 16 Mar 2006 (16.03.2006); para [0005], [0018], [0055]	8-10, 17-18
Y	US 5,800,467 A (PARK et al.) 01 Sep 1998 (01.09.1998); col 7, ln 1-15	11-14, 20-23
Y	US 5,725,563 A (KLOTZ) 10 Mar 1998 (10.03.1998); col 5, ln 30-38; col 6, ln 26-34	31-33
Y	US 7,294,334 B1 (MICHAL et al.) 13 Nov 2007 (13.11.2007); col 13, ln 4-12; col 19, ln 46-50; col 30, ln 65-col 31 ln 3	35-37

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

11 June 2009 (11.06.2009)

Date of mailing of the international search report

24 JUN 2009

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Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Lee W. Young

PCT Helpdesk: 571-272-4300

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International Bureau

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11 November 2010 (11.11.2010)



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61/310,557 4 March 2010 (04.03.2010) US
61/323,782 13 April 2010 (13.04.2010) US

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(72) Inventors; and

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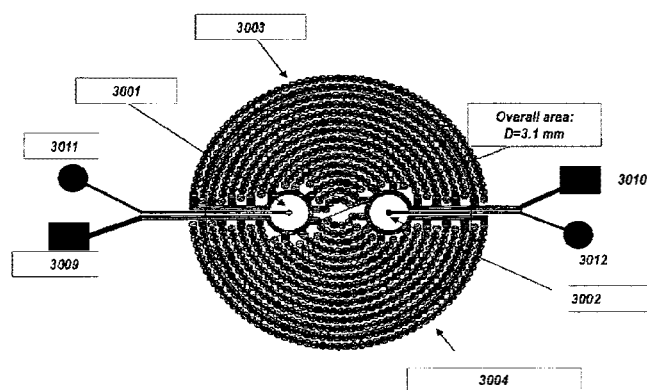


Figure 30

(57) Abstract: Devices and methods for performing extracellular recording of cells, such as excitable cells, cardiomyocytes, and cardiomyocyte precursor cells is provided. An exemplary device includes a nonconductive substrate forming or provided as a base of one or more wells; a recording electrode positioned on the substrate within the well, wherein the recording electrode is accessible to cells when a cell sample is added to the device; and a reference electrode positioned within the well in a cell-free zone, the cell-free zone characterized as free from contact with cells when the cell sample is added to the device, thereby preventing contact between cells and the reference electrode.

**SYSTEM AND METHOD FOR MONITORING CARDIOMYOCYTE
BEATING, VIABILITY AND MORPHOLOGY AND FOR SCREENING FOR
PHARMACOLOGICAL AGENTS WHICH MAY INDUCE
CARDIOTOXICITY OR MODULATE CARDIOMYOCYTE FUNCTION**

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. patent application serial no. 61/323,782, filed on April 13, 2010, entitled, "Impedance based monitoring of cardiomyocytes"; U.S. patent application serial no. 61/310,557, filed March 15, 2010, entitled, "Impedance based monitoring of cardiomyocytes"; and U.S. patent application serial no. 61/175,566, filed May 5, 2009, entitled, "System and method for monitoring cardiomyocyte beating, viability and morphology and for screening for pharmacological agents which may induce cardiotoxicity or modulate cardiomyocyte function." The contents of each are herein incorporated by reference in their entirety.

TECHNICAL FIELD

This invention relates to the field of cell-based assays and more specifically to devices, methods and systems for performing extracellular recording of cells, such as cardiomyocytes, alone or in parallel with impedance monitoring.

BACKGROUND OF THE INVENTION

A. Electronic Analysis of Cells

Bioelectronics is a progressing interdisciplinary research field that involves the integration of biomaterials with electronic devices. Bioelectronics methods have been used for analyzing cells and assaying biological molecules and cells. In one type of application, cells are cultured on microelectrodes and cell-electrode impedance is measured and determined to monitor cellular changes.

In PCT Application No. PCT/US03/22557, titled "IMPEDANCE BASED DEVICES AND METHODS FOR USE IN ASSAYS", filed on July 18, 2003, a device for

detecting cells and/or molecules on an electrode surface is disclosed. The device detects cells and/or molecules through measurement of impedance changes resulting from the attachment or binding of cells and/or molecules to the electrode surfaces. A number of embodiments of the device are disclosed, together with the apparatuses, and systems for using such devices to perform certain cell based assays.

B. Dynamic Monitoring of Cardiomyocyte Viability, morphology and Beating In Vitro

To bring a new drug to the market it can take anywhere between 8 to 16 years, and average cost of developing a drug is now around \$500m-\$800m with the cost expected to hit the \$1bn mark within the next four years. Cardiotoxicity has been cited as the reason for 30 per cent of all failed drug compounds during development and is a major cause of compound attrition. The late detection of cardiotoxic side effects caused by pharmacological compounds can impede drug discovery and development projects, and consequently increase their cost. Testing for the potential cardiotoxic side effects of compounds at an early stage of drug development has therefore been the goal of many pharmaceutical and biotechnology companies. Cardiotoxicity itself can entail a number of short-term and long term cellular events including directly affecting the beating rate of cardiomyocytes, viability of cardiomyocytes and morphology of cardiomyocytes as would occur in hypertrophy. The core of the current issue in pharmacological safety assessment and drug development is the lack of a reliable screening methodology capable of monitoring potential drug-mediated cardiotoxicity and distinguishing between different modes of cardiotoxicity. What is urgently needed in the field is a good cell-based model system as well as a monitoring system with a physiological and functional readout that can provide incisive information regarding potential cardiotoxic side effects of drugs.

1. Cell-based Model System for Studying Cardiac Function

Traditionally, the drug discovery industry has undertaken two different approaches for toxicological assessment of drug candidate leads in cardiac function. The first approach involves isolation of cardiomyocytes directly from a mammalian species such as rats and dogs followed by electrophysiological and viability studies on the

isolated cardiomyocytes. This approach is extremely labor-intensive, time consuming and costly and at the same time not very amenable to the high throughput demands of pharmaceutical industry.

5 An alternative method for prediction of cardiotoxicity of drug candidate leads early in the drug development process has involved utilizing cell-based assay models which heterologously express specific ion channels such as hERG channels or voltage-gated calcium channels. These cardiac ion channels have been envisioned as possible molecular targets through which drugs could induce cardiotoxicity. These cell-based
10 systems allow the assessment of drug-channel interaction by monitoring the effect of the drug on the currents produced by the different channels in cultured cells using a technique known as 'patch clamping', which isolates regions of the cell membrane containing channel proteins and measures changes in electrical potential difference. Use of this method in high throughput requires automation of patch clamping in array
15 format, which even though is available in last several years but is not yet widespread. The other issue with this approach is that cardiac toxicity may occur by other mechanisms which can easily be missed by this type of targeted approach. An alternative to the *in vitro* ion-channel recording assays as well as the labor-intensive isolation of primary tissue is the differentiation of embryonic stem (ES) cells into
20 cardiomyocytes as a starting material for functional assays.

The utility of ES cells as a treatment for various chronic diseases has received much attention in recent years. Mammalian ES cells are self renewing cells derived from the inner cell mass of a blastocyst stage embryo which can be differentiated into multiple
25 different cell types. It has been demonstrated that the mouse ES cells as well as human ES cells can be differentiated into cardiomyocytes which retain the ability to beat in culture. The differentiation of ES cells first involves an intermediate *in vitro* developmental stage in which ES cells form compact cell structures known as embryoid bodies. These embryoid bodies can induce the developmental program of
30 ES cell differentiation into multiple cell types including cardiomyocytes which are distinguished in culture by their ability to undergo spontaneous beating. These ES derived *in vitro* differentiated cardiomyocytes recapitulates the normal development of cardiomyocytes as evidenced by the stage-specific expression of cardiomyocyte specific genes. All the known transcription factors, ion channels and structural

proteins that are part of normal heart development and function *in vivo* are also expressed in ES-derived cardiomyocyte.

Because ES cells are self renewing cells in culture they can serve as an excellent
5 source for continuous production of cardiomyocytes. Therefore, these cardiomyocytes
which behave in every way like normal cardiomyocytes isolated from the heart tissue
itself addresses the ever important supply problem and for the first time allows for
assessment of cardiac function and its modulation by lead candidate drugs and
compounds in relatively large scale in both viability assays, assessment of
10 morphology and in monitoring the beating function of cardiomyocytes. Furthermore,
because the technology exists to selectively knockout or express trans-genes in ES
cells, it provides an excellent model system to study the role of certain genes in
cardiac development and function without having to be concerned about adverse
affects on overall embryonic development in transgenic animals. The ability to
15 express transgenes in ES cells has been utilized as a way to enrich for preparation of
cardiomyocytes that are 100% pure. For example, the gene encoding GFP has been
cloned downstream of a cardiac-specific promoter and then introduced into ES cells.
Embryoid cells which ultimately differentiate into cardiomyocytes will therefore
express the GFP transgenes and these cells can be easily isolated by cell sorting
20 techniques and therefore an enriched cardiomyocyte population can be obtained.

2. Technologies Used for Assessment of Cardiomyocyte Function *In Vitro*

Technologies designed to assess cardiomyocyte behavior and function and the effect
25 of drugs and other manipulations *in vitro* can be divided into two different
approaches. One approach involves long term assessment of cardiomyocyte viability
for example in response to certain compounds. Such assays are typically end point
assays designed to measure a cellular component such as ATP which correlates with
the degree of viability of the cells. The other approach involves studying short term
30 effect of drugs and compounds on beating function of cardiomyocytes. High
throughput techniques for short term functional characterization of ion channels and
other targets in cardiomyocytes has been rather challenging and limited. The systems
such as automatic patch clamp instrumentation that are available can monitor a single
cardiomyocyte at a time and with very limited throughput.

Accordingly, while there exists different approaches to assess cardiotoxicity of various compounds, each suffers from one or more drawbacks. Thus, there remains a need to develop improved devices and methods that have high throughput capabilities and improved accuracy and reliability with respect to measurements and correlation with cell phenotype.

SUMMARY OF THE INVENTION

10 The present invention discloses devices, systems and methods for performing extracellular recording of excitable cells, such as cardiomyocytes, *in vitro*. Specifically, the devices and methods provide improved characterization of excitable cells in response to compound administration to assess cardiotoxic effects, while increasing efficiency of throughput for potential drug candidates. The invention
15 further provides improved characterization of cells during differentiation processes related to development of cardiomyocytes.

It is an object of the invention to provide extracellular recording configurations, which permit reproducible sampling across larger cell populations compared to
20 traditional approaches. It is another object of the invention to provide cell-free zones for positioning a reference electrode to prevent its direct interaction with a cell population loaded onto the device. It is another object of the invention to provide a combined device capable of both performing extracellular recording measurements and impedance monitoring, which may be selectively activated for measurement and
25 thus provide a multi-faceted system for assessing or characterizing excitable cells.

In one aspect of the present invention a device for performing extracellular recording of cells, such as excitable cells, cardiomyocytes, and cardiomyocyte precursor cells is provided. An exemplary device includes a) a nonconductive substrate forming or
30 provided as a base of one or more wells; b) a recording electrode positioned on the substrate within the well, wherein the recording electrode is accessible to cells when a cell sample is added to the device; and c) a reference electrode positioned within the

well in a cell-free zone, the cell-free zone characterized as free from contact with cells when the cell sample is added to the device, thereby preventing contact between cells and the reference electrode. The cell-free zone prevents direct contact between cells from a cell sample and the reference electrode. In some embodiments, the recording
5 electrode has a diameter from about 10 μm to about 200 μm . In other embodiments, the recording electrode comprises an electrode structure comprising a plurality of electrode elements

In some embodiments, the cell-free zone is physically defined by the presence of a
10 barrier, such as a wall, through which cells do not pass. In certain embodiments, the barrier could be in the form of a removable blockage material which is placed directed on the cell-free zone when cells are added to the well and which can be removed after cells have settled along the bottom of the well. Thus, the blocking structure may be in part removable. In other embodiments, the cell free zone is provided as a platform or
15 surface positioned above the base substrate. In other embodiments, the cell free zone is positioned along an angled wall, which is angled upward from the bottom of the well. In other embodiments, the cell-free zone is positioned above the substrate and within the volume of the well such that an external electrode extends downward into sample media, optionally from a lid or cap for the well.

20 In some embodiments the extracellular recording electrode is a unitary or unbranched electrode and may be of a simple geometry such as a circle, a square and the like. In other embodiments, the extracellular recording electrode has a branched configuration, which may permit extracellular recording over a larger cell population.
25 Increasing the measured cell population for extracellular recording may increase measurement accuracy or reproducibility of the recorded signals. In one exemplary embodiment, a device for performing extracellular recordings of excitable cells in vitro includes a) a nonconductive substrate; b) one or more wells on the substrate; c) one or more electrode structures fabricated on the substrate, each electrode structure
30 associated with one of the one or more wells; and d) one or more reference electrodes external to the substrate, each of which can be inserted into one of the one or more wells, wherein for each of the one or wells, the corresponding electrode structures and reference electrodes form an electrode pair. In further embodiments the substrate has a surface suitable for attachment of excitable cells, wherein the attachment of

excitable cells on the substrate can result in a detectable extracellular recording potential between each electrode pair. In a further embodiment, which is also a preferred embodiment, each electrode structure comprises multiple electrode elements. In another preferred embodiment, each electrode structure comprises multiple electrode elements, forming half of an interdigitated electrode array. In another preferred embodiment, each electrode structure comprises multiple electrode elements which have different electrode element shapes, including rectangular shape, or sinusoidal shape, or circle-on-line shape.

- 10 In another preferred embodiment of the device for performing extracellular recording, each electrode structure occupies a substantial percentage of surface area of the well that the electrode structure is associated with. In some preferred embodiments, percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 1%. Preferably, percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 5%. Preferably, percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 10%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 20%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 30%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 50%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 70%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 85%.

- 30 In another aspect of the present invention, a method of performing extracellular recording of excitable cells includes, providing a device of the present invention, adding excitable cells to the wells; providing an extra-cellular-recording amplifier that can measure and record voltage signals at microvolt levels, connecting electrodes on the devices to the extra-cellular-recording amplifier; monitoring and recording extracellular potentials at the electrodes of the devices. Preferably, the method further comprises analyzing recorded extracellular potential waveforms. More preferably, the method further comprises adding a compound to the well, measuring

and recording extracellular potentials prior to and after the compound addition; and analyzing extracellular potential waveforms.

In still another aspect of the present invention, a device allowing for parallel
5 extracellular recording and cell impedance monitoring of a cell population is provided. The device includes a cell impedance monitoring means and an extracellular recording means operably coupled through a same substrate. In a further embodiment a switching means switches operation of the device between impedance monitoring and extracellular recording.

10

In still another aspect of the present invention, a device allowing for parallel extracellular recording and cell impedance measurement comprises a nonconductive substrate, having a surface suitable for attachment of excitable cells, one or more wells on the substrate; for each well, a pair of impedance measurement electrodes,
15 wherein the attachment of excitable cells on the substrate can result in a detectable impedance change between the pair of impedance measurement electrodes; for each well, a pair of extra-cellular recording electrodes, wherein the attachment of excitable cells on the substrate can result in a detectable extra-cellular recording potentials between the pair of extra-cellular recording electrodes. Preferably, the pair of
20 impedance measurement electrodes is located on the substrate. More preferably, for the pair of extra-cellular recording electrodes, one recording electrode is located on the substrate and another electrode is located external to the substrate. In other preferred embodiments, the reference electrode may be isolated from cells by positioning the reference electrode at a cell-free zone, such that it is a cell-free
25 electrode. The reference electrode may or may not be located on the substrate. The device may also include or be operably connected to a switching means to switch from an impedance mode to an extracellular recording mode.

In another aspect of the invention, a device allowing for parallel extracellular
30 recording and cell impedance measurement includes a nonconductive substrate forming or provided as a base of one or more wells; at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate; and a reference electrode, wherein a first impedance electrode from the at least two impedance

electrodes is electrically coupled to the reference electrode for performing an extracellular recording measurement at the first impedance electrode.

5 In still another aspect of the present invention, a method for parallel measurement of cell-substrate impedance and extra-cellular potentials includes a) providing a device allowing for parallel extracellular recording and cell impedance measurement; b) adding excitable cells to the wells of the device; c) providing an impedance analyzer; d) providing an extracellular potential amplifier; e) connecting impedance-measurement electrodes of the devices to the the impedance analyzer; f) connecting
10 extra-cellular recording electrodes of the devices to the the extra-cellular potential amplifier; g) performing parallel measurement of cell-substrate impedance and extracellular potentials. Preferably, the method further comprises adding compounds to the cells and monitoring cell-substrate impedance and extracellular potentials prior to and after compound addition. Preferably, the method further comprises analyzing
15 measured time dependent impedance responses. Still preferably, the method further comprising analyzing extracellular-recording wave forms.

In present invention, we describe label-free methods for monitoring cardiomyocytes in vitro. In one aspect of the present invention, extracellular-recording
20 measurement, or parallel impedance and extracellular recording measurement of cardiomyocytes on microelectrodes is used to non-invasively monitor cardiac cell viability in vitro in long term experiments. This method will allow the continuous monitoring of cardiomyocyte viability overtime and can monitor the interaction of compounds which ultimately result in promoting loss of cardiomyocyte viability.

25 In another aspect of the present invention, extracellular-recording measurement or parallel impedance and extracellular recording measurement of cardiomyocytes on microelectrodes is used to non-invasively monitor cardiomyocyte beating *in vitro*. The system is capable of continuously monitoring the excitation-contraction coupling,
30 otherwise know as beating, of cardiomyocytes in a relatively high-throughput manner using either extracellular-recording measurement or parallel impedance and extracellular recording measurement. It can be used for pharmacological safety assessment, screening for novel compounds which modulate cardiomyocyte function in a specific manner and assessment of genes involved in cardiac function.

In another aspect of the present invention, extracellular recording measurement or parallel impedance and extracellular recording measurement can be used to monitor both short term beating whilst impedance measurement can be used to monitor long term viability status of cardiomyocytes in the same well. The ability to monitor both cardiomyocyte beating in conjunction with viability for the same population of cardiomyocytes over time would be an added advantage. Certain manipulations such as drug treatment may not manifest their effect on cardiomyocyte beating and/or viability until at a later time period. Therefore, the time resolution as well as the capabilities of extracellular recording measurements together with impedance measurements of the system of the present invention can be used to monitor cardiomyocyte beating and viability over time.

In another aspect of the present invention, impedance readout can be used to monitor the morphological or differentiative behavior of cardiomyocytes *in vitro*. Certain treatments can induce changes in morphological behavior of cardiomyocytes, such as inducing hypertrophy which is associated with cardiomyocyte elongation and expansion. Because impedance monitoring can detect changes in cell morphology, it can be used to for detection of hypertrophy in cardiomyocytes.

In another aspect of the present invention, direct optical monitoring of cardiomyocytes is used to quantify and measure the beating of cardiomyocytes. Still, in another aspect of the present invention, extracellular recordings (i.e. measurement of electrical potentials of extracellular recording electrodes when cardiomyocytes are attached to the electrode surfaces) are used to quantify and measure the beating of cardiomyocytes. Still, in another aspect of the present invention, a physical method which can be used to monitor cell-substrate interaction is used to quantify and monitor beating of cardiomyocytes.

In another aspect of the present invention, the methods include real-time impedance monitoring and/or extracellular recording of cardiomyocyte *in vitro*. This invention will be able to monitor viability of cardiomyocytes, the field potential of cardiomyocytes, or the excitation-contraction coupling or beating and morphological

and differentiative aspects of cardiomyocytes in a label-free manner and real-time manner. Furthermore, the invention will be able to monitor cardiotoxicity and the effect of compounds and drugs for safety pharmacology purposes and can also serve as a screen for agents which can modulate cardiomyocyte function. This system can
5 be used in conjunction with ES derived cardiomyocytes, adult stem-cell derived cardiomyocytes as well as primary cardiomyocytes to study the role of different genes and proteins in cardiac function and development.

The system of the present invention having the capability of parallel impedance-based
10 monitoring as well as extra-cellular recording based monitoring of cardiomyocytes fills a major technological gap in monitoring cardiomyocytes *in vitro*. At present to our knowledge there are only a few technologies that can monitor cardiomyocyte population function *in vitro*, especially with regards to cardiotoxicity and are limited in their throughput. In addition to functional monitoring of cardiomyocyte beating the
15 current invention offers several other major benefits which are worth discussing. Among these include the impedance system described here can monitor cardiomyocytes for short durations, milliseconds to minutes and long durations, several hours to days. Therefore, both short term and long term effects of drugs not only on cardiomyocyte beating, but viability and changes in morphology and adhesion
20 can also be assessed. This feature is especially important because certain compounds such as β -2 adrenergic receptor agonists, well known and characterized modulators of heart function *in vivo* and *in vitro*, can induce long term hypertrophic responses in cardiomyocytes, which is associated with elongated morphology of the cells. In addition, the systems of the present invention can also record cardiomyocyte action
25 potentials or field potentials, allowing detailed electrophysiological analysis and assessment of cardiomyocytes for relatively short duration at a time.

The method of the present invention is to devise a label-free cell-based assay system for continuous monitoring of cardiomyocyte viability, the rhythmic beating of
30 cardiomyocytes and cardiomyocyte morphology and differentiation and to screen for pharmacological agents which may modulate these processes and induce cardiotoxicity. In one aspect, viability is monitored based on long term impedance monitoring of cardiomyocytes seeded in microelectronic plates (E-PLATES), and in

parallel, the field potential generated by the excitation of the cardiomyocytes or other excitable cells is monitored using the extracellular potential recording electrodes associated with the wells of the E-PLATES. Viable cells will continue to generate impedance signal and any changes in viability, especially due to cytotoxic or cardiotoxic drugs will be reflected by changes in impedance. In one respect, the method includes providing a device for measuring cell-substrate impedance and extracellular potentials operably connected to an impedance analyzer and extracellular recording potential amplifier, wherein the device includes at least one well optionally coated with fibronectin or other appropriate extracellular matrix proteins (e.g. gelatin) to expedite attachment; adding cells to the at least one well, where the cells can be mouse or human or other mammalian ES cells or adult stem cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from the heart of an experimental system including mice, rats, rabbits or dog; monitoring impedance of the at least one well over a period of time and optionally determining cell index from impedance values; and monitoring the extracellular potentials using recording electrodes associated with the wells of the E-PLATES.

In another aspect, the method includes providing a device for measuring cell-substrate impedance and for performing extracellular recording operably connected to an impedance analyzer and extracellular recording potential amplifier, wherein the device includes at least two wells optionally coated with fibronectin (or other extracellular matrix proteins such as gelatin) to expedite attachment; adding cells to the at least two wells, where the cells can be mouse or human or other mammalian ES cells or adult stem cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from the heart of an experimental system including mice, rats, rabbits or dog; monitoring impedance and monitoring extracellular potentials of the at least two wells over a period of time. Treating at least one well with an agent; where the agent could include but is not limited to a compound, peptide, protein, antibody, siRNA, shRNA, lipid or any combination of thereof and the other well is treated with an appropriate control; continue monitoring of control and treated well over a period of time preoptimized for the experiment of interest; concluding that the factor may affect cell viability if the impedance of the treated well is significantly different than the impedance of the treated well and/or concluding that

the factor may affect cell excitation if the extracellular potential of the treated well is significantly different from that of the treated well.

Dynamic monitoring of cardiomyocyte beating is based on quantification in real time
5 of the rhythmic changes in cardiomyocyte morphology as a result of the excitation
contraction coupling of the electrically excitable cardiomyocytes growing on
microelectrodes' surface in ACEA E-PLATES. The quantification of the rhythmic
changes in cardiomyocyte morphology is achieved via the fast (down to milliseconds
level, for example, at 40 milli-second, 30 milli-second, 20 milli-second, 10
10 millisecond or 5 milli-second time resolutions) and continuous measurement of
electrode impedance. The method essentially provides a cellular cardio-gram which
can provide incisive information about the status of cardiomyocytes especially upon
treatment with pharmacological agents. In addition, the method allows for parallel
measurement of extracellular potentials of beating cardiomyocytes, providing
15 important electro-physiological property information of the cells. The method
includes providing a device for measuring cell-substrate impedance and for
monitoring extracellular potential operably connected to an impedance analyzer and
an extracellular potential amplifier, wherein the device includes at least one well
optionally coated with fibronectin (or other suitable extracellular matrix proteins) to
20 expedite attachment; adding cells to the at least one well, where the cells can be
mouse or human or other mammalian ES cells or adult stem cells destined to
differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from
the heart of an experimental system including mice, rats, rabbits or dog; monitoring
impedance of the at least one well at time intervals over a period of time and
25 optionally determining cell indices from impedance values and monitoring
extracellular potentials from the at least one well for a time period (for example, 10
seconds) at certain time intervals (for example, every 10 minutes); optionally
calculating average rate of beats per unit time, average amplitude intensity in a unit
time as well as the average length of time between the beats.

30 In another aspect, the present invention is directed to method to screen for potential
agents that modulate ES- derived cardiomyocyte beating, adult stem cell-derived
cardiomyocyte beating or primary cardiomyocyte beating by monitoring and
measuring the excitation-contraction coupling of cardiomyocyte and its modulation

by the agent. The agent may include but is not limited to compounds, drugs, peptides, proteins, antibodies, siRNA, shRNA, miRNA, cDNA, lipids and any combination thereof. The method includes providing a device for measuring cell-substrate impedance and for monitoring extracellular potentials operably connected to an impedance analyzer and to an extracellular potential amplifier, wherein the device includes at least two wells; adding ES cells, adult stem-cell derived cardiomyocytes or primary cardiomyocytes to at least two wells; monitoring impedance of the at least two wells at different or similar time intervals over a period of time and optionally determining cell indices from impedance values and monitoring and recording extracellular potentials of the at least two wells for a time window at time intervals over a period of time; generating an impedance-based curve or optionally a cell index curve for each of the at least one known factor and the control; comparing the impedance-based curves or optionally the cell index curves between the at least one known biologically active agent well and the control well; the impedance-based curves could be direct measurement of cardiomyocyte excitation-contraction coupling and if significantly different, concluding that the biologically active agent modulates cardiomyocyte function, and/or comparing the extracellular potential waveforms from the active agent well and the control well and, if significantly different, concluding that the biologically active agent modulates the excitation property of the cardiomyocytes. Optionally, impedance-based curves or optionally cell index curves are used to calculate average rate of beats of cardiomyocytes per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats, comparison of these optionally derived parameters is made between the at least one known biologically active agent well and the control well, and if significant differences exist, one may optionally conclude that the biologically active agents modulate cardiomyocyte functions.

It is well established that certain pharmacological treatments and disease conditions can result in cardiac hypertrophy or atrophy culminating in changes in the morphology of cardiomyocyte. Cell substrate impedance can be used to precisely measure and quantify these changes in cell morphology and shape. Certain treatments can also affect the differentiative process of ES cells to cardiomyocytes which may involve specific morphological and adhesive changes. In another aspect, the present invention is directed to method to screen for potential agents that may modulate the

morphology of ES- derived cardiomyocyte, adult stem cell-derived cardiomyocyte or primary cardiomyocyte or its differentiation. The agent may include but is not limited to compounds, drugs, peptides, proteins, antibodies, siRNA, shRNA, miRNA, cDNA, lipids and any combination thereof. The method includes providing a device for

5 measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least two wells; adding ES cells, adult stem-cell derived cardiomyocytes or primary cardiomyocytes to at least two wells; monitoring impedance of the at least two wells at different or similar time intervals over a period of time and optionally determining cell indices from impedance values; generating an
10 impedance-based curve or optionally a cell index curve for each of the at least one known factor and the control; comparing the impedance-based curves or optionally the cell index curves between the at least one known biologically active agent well and the control well; the impedance-based curves could be direct measurement of changes in cell morphology and if significantly different, concluding that the
15 biologically active agent modulates cardiomyocyte function. Preferably, the method further comprises providing the device of the present invention operably connected to an extracellular potential amplifier, monitoring extracellular potentials of cells from one biologically active agent well and one control well. Optionally, impedance-based curves or optionally cell index curves are used to calculate the compound dose-
20 dependent changes in cardiomyocyte morphology and generate an EC-50 value for the potency of the compound. In addition, extracellular recording permits comparisons with cells at various developmental stages to assess development.

In another aspect, the present invention is directed to method to establish an assay to
25 assess the effect of gene knockout or transgene expression in ES cells differentiated to cardiomyocytes and functionally monitored by the system of the present invention.

The method includes providing a device for measuring cell-substrate impedance and for monitoring extracellular potential operably connected to an impedance analyzer and extracellular potential amplifier, wherein the device includes at least two wells;

30 adding wildtype ES cells as control to at least 1 well and ES cells with a gene knockout or a transgene in at least 1 other well; monitoring impedance of the at least two wells at time intervals over a period of time and optionally determining cell indices from impedance values; comparing the impedance-based curves or optionally the cell index curves between the control well and the well containing the ES cells

harboring a knockout of a specific gene or expressing a specific transgene; and if significantly different, concluding that the gene knockout or the transgene can affect either cardiomyocyte viability, morphology from ES cells or cardiomyocyte function as monitored by observing the excitation-contraction coupling, and alternatively,
5 monitoring and recording extracellular potentials from the at least two wells for a time period, comparing the extracellular potential waveforms between the control well and the well containing the ES cells harboring a knockout of a specific gene or expressing a specific transgene; and if significantly different, concluding that the gene knockout or the transgene can affect cardiomyocyte electrophysiological property of the
10 cardiomyocytes.

In another aspect of the present invention, direct optical monitoring of cardiomyocytes is used to quantify and measure the beating of cardiomyocytes. The method includes providing a device for optically monitoring cells and monitoring cell
15 morphology operably connected to an optical measurement system, where the device includes at least two wells optionally coated with fibronectin (or other suitable extracellular matrix proteins) to expedite attachment; adding cells to the at least two wells, where the cells can be mouse or human or other mammalian ES cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from
20 the heart of an experimental system including mice, rats, rabbits or dog; optically monitoring the cells of at least two wells at time intervals over a period of time via the optical measurement system; optionally calculating average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats. The device for such optical measurement of cells may include
25 microtiter plates. The optical system may include optical magnification instrument such as microscope, optical CCD camera, optical-signal processing algorithm to quantify cell beating and to derive cell-beating parameters (such as calculating average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats) based on cell morphology images.

30 In another aspect of the present invention, extracellular recordings (i.e. measurement of electrical potentials of extracellular recording electrodes when cardiomyocytes are attached to the electrode surfaces) are used to quantify and measure the beating of cardiomyocytes. The method includes providing a device with microelectrodes for

extracellular recording of multiple cells operably connected to an extracellular potential recording system, where the device includes at least two wells optionally coated with fibronectin (or other extracellular matrix proteins) to expedite attachment; adding cells to the at least two wells, where the cells can be mouse or human or other mammalian ES cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from the heart of an experimental system including mice, rats, rabbits or dog; performing extracellular recording of the cells of at least two wells via the extracellular recording system; optionally calculating average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats. The device for such extracellular recording system includes microelectrode arrays or structures inside wells of microtiter plates. The microelectrode array may comprise two electrode structures having the substantially same surface area and may be located along the majority of the surface of the wells. The extracellular recording system may include electronic measurement circuits capable of recording small extracellular potentials induced on microelectrodes, where cells are cultured. The signals from the extracellular recording can be used to derive the average rate of beats of cardiomyocytes per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats, or other electrophysiologically-associated parameters.

Still, in another aspect of the present invention, a physical method which can be used to monitor cell-substrate interaction is used to quantify and monitor beating of cardiomyocytes. The method includes providing a device for physically monitoring cells and monitoring cell morphology operably connected to a physical measurement system, where the device includes at least two wells optionally coated with fibronectin (or other extracellular matrix proteins) to expedite attachment; adding cells to the at least two wells, where the cells can be mouse or human or other mammalian ES cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from the heart of an experimental system including mice, rats, rabbits or dog; optically monitoring the cells of at least two wells at time intervals over a period of time via the physical measurement system; optionally calculating average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats. The device for such physical measurement of cells shall have such property that the interaction between

cells and substrate surfaces can be monitored. Depending which physical method is used for monitoring cell-substrate interaction, different devices may be used. For example, the device can be an optical sensor, which can be used to detect and measure biological reactions. One example of optical sensor is resonant waveguide, which consists of a substrate with an optical grating and a coating with a high refraction index. This grating forms an optical waveguide, a tiny channel through which a specific wavelength of light propagates. Only this specific wavelength that is resonant with the waveguide grating structure is strongly reflected. In this case, the physical measurement system can detect changes in refractive index upon cell-substrate interaction events or upon intracellular events. Examples of cell-substrate interaction events or intracellular events may include those taking place in cultured cells stimulated by a compound treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cell index curves measured on RT-CES system for 4 different seeding densities (3750, 7500, 15,000 and 30,000 cells per well) of mouse ES-derived cardiomyocytes. Cell index is a dimensionless parameter derived in RT-CES system.

Figure 2A shows the time dependent, normalized cell index for embryonic stem cell derived cardiomyocytes in different wells treated with different concentrations of sodium dichromate dehydrate. **Figure 2B** shows the dose dependent normalized cell index values at 24 hrs after treatment of the cells with dichromate dehydrate as a function of the concentration of dichromate dehydrate.

Figure 3A shows that the time dependent, normalized cell index for embryonic stem cell derived cardiomyocytes in different wells treated with different concentrations of isoproteranol. **Figure 3B** shows the dose dependent, normalized cell index at several hours after treatment of the cells with isoproteranol as a function of the concentration of isoproteranol.

Figure 4 shows the cell index curves of ES-derived cardiomyocytes measured on RT-CES system for 4 individual wells, with cell culture medium as background starting from cell seeding to about 86 hrs after cell seeding.

Figure 5 shows the detection of cardiomyocyte beating using the ACEA RT-CES system at different time periods along the growth and differentiative curve of ES-derived cardiomyocytes seeded in ACEA E-PLATES. Each well of the E-PLATE
5 comprises an impedance measurement electrode array, comprising two circle-on-line electrode structures. **Figure 5A, 5B and 5C** show the cell index curves at time resolution of 40 milli-seconds for time points at 24, 48 and 72 hrs after cell seeding, respectively. **Figure 5D** shows the cell index curves monitored at 1 hr time resolution.

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Figure 6A and 6B show the cell index curve for ES-derived cardiomyocytes and corresponding beat rate as analyzed using Fourier transform.

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Figure 7 shows the detection of cardiomyocyte beating using the ACEA RT-CES system for the mouse ES-derived cardiomyocytes treated with 4.4 uM sotalol.

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Figure 7A and 7B show the beating rate and amplitude, respectively, for the cells before and after the treatment. **Figure 7C** shows overall cell index curves whilst **Figure 7D** shows the cell-index based beating curve for a short time period. Note that one second displayed in **Figure 7C and 7D** is equivalent to 40 milli-seconds for real time measurement. The time resolution of the RT-CES measurement for **Figures 7C and 7D** is 40 milli-seconds.

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Figure 8A and 8B show the detection of cardiomyocyte beating using the ACEA RT-CES system to assess the effect of chemical compound carbachol (333 nM, **8A**) and isoproterenol (4.4 uM, **8B**) on the beating of cardiomyocyte. Note that one second displayed in **Figure 8A and 8B** is equivalent to 40 milli-seconds for real time measurement. The time resolution of the RT-CES measurement for **Figures 8A and 8B** is 40 milli-seconds.

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Figures 9A-S show the detection of cardiomyocyte beating using the ACEA RT-CES system to assess the effect of various chemical compounds on the beating of mouse ES-derived cardiomyocytes: (**9A**) 33.3 uM sotalol with 0.033% DMSO; (**9B**) 400 nM Astemizole with 0.004% DMSO; (**9C**) 200 nM terfenadine with 0.065% DMSO; (**9D**) 13.3 uM Erythromycin with 0.13% DMSO; (**9E**) 20 uM moxifloxacin with 0.3%

DMSO; **(9F)** 20 uM pentamidine with 0.2% DMSO; **(9G)** 4.4 uM amitriptyline with 0.04% DMSO; **(9H)** 130 nM verapamil with 0.0013% DMSO; **(9I)** 13.3 uM rosiglitazone with 0.13% DMSO; **(9J)** 13.3 uM rofecoxib; **(9K)** 4.4 uM celecoxib; **(9L)** 40 uM doxorubicin with 0.4% DMSO; **(9M)** 13.3 uM cyclosporine with 0.13% DMSO; **(9N)** 4.4 uM propranolol with 0.04% DMSO; **(9O)** 9.1 nM E4031; **(9P)** 8 uM DDT with 0.08% DMSO; **(9Q)** 8uM PCB with 0.08% DMSO; **(9R)** 8uM endosulfan with 0.08% DMSO; **(9S)** 0.13% DMSO. **Figure 9S** shows the 0.13% DMSO control, indicating that DMSO at such a concentration does not produce a significant change on the beating of the cardiomyocytes. Note that one second displayed in **Figures 9A – 9S** is equivalent to 40 milli-seconds for real time measurement. The time resolution of the measurement for **Figures 9A – 9S** is 40 milli-seconds.

Figure 10 shows the change of the beating pattern of mouse ES-derived cardiomyocytes as a result of treatment with different compounds as monitored by ACEA RT-CES system. The compounds being tested here include 130 nM verapamil, 40 nM astemizole, 20 uM sotalol; 200 nM trefenadine; 4 uM propranolol; 4 uM celexocib; 150 nM carbachol and 82 nM E4031.

Figure 11 shows the change of the beating pattern of mouse ES-derived cardiomyocytes as a result of treatment with compound Aztemizole as monitored by ACEA RT-CES system. The compounds concentrations being tested here include 400 nM, 40 nM, 13.3 nM, 1.33 nM, 0.133 nM and 40 pM.

Figure 12 shows a pair of extracellular recording electrodes, comprising a recording electrode structure on the substrate and a reference electrode external to the substrate.

Figure 13 shows examples of electrode structures having multiple electrode elements that can be used for extracellular recording and/or impedance measurement where **13A, 13B, 13C, 13D, 13E** and **13F** are the rectangular shape, circle-on-line shape, a complete interdigitated electrode array, sinuosoidal shape, castellated shapes and a complete circle-on-line electrode array, respectively.

Figures 14A and 14B show two examples of extracellular recording devices where each well comprises one recording electrode structure comprising multiple electrode elements and a reference electrode having large surface area.

5 **Figure 15** shows an example of an extracellular recording device where each well of the device comprises a simple, circle-shaped recording electrode and a reference electrode (**1501**) having large surface area (**1502**).

10 **Figure 16** shows an example of a device of the present invention where in one well, a pair of impedance measurement electrodes is located on the substrate and one of the impedance measurement electrode structures is used as an extracellular recording electrode, together with an externally applied reference electrode.

15 **Figure 17** shows a multi-electrode chip comprising an array of 30 electrodes where the electrode diameter is 50 μm and the interval between each two neighbor electrode tips is 50 μm .

20 **Figure 18** shows the extracellular field potential (FP) recorded for mouse ES-derived cardiomyocytes on day 2 and day 3 after cell plating using electrode array shown in **Figure 17**.

Figure 19 shows the extracellular field potential (FP) recorded for mouse ES-derived cardiomyocytes on day 3 after cell plating using electrode array shown in **Figure 17**. The cells are treated with compound E4031 at different concentrations ranging from 0 nM (control), 75 nM, 150 nM, 250 nM and 750 nM respectively.

Figure 20 shows the dependency of field potential duration in milli-seconds on the concentration of E4031.

30 **Figure 21** shows the “anti-arrhythmic” effect of compound E4031 at 75 nM and 150 nM on field potential frequency for the mouse ES-cell derived cardiomyocytes.

Figure 22 shows extra-cellular field potentials recorded for mouse stem-cell derived cardiomyocytes obtained using a device of the present invention, where the recording

electrode is a circle-on-line electrode structure and the reference electrode is a gold wire electrode that is introduced into the well after cell seeding. **Figure 22A, 22B, 22C and 22D** shows the recorded waveforms before treatment, ~ 18 seconds, ~ 3 minutes and ~ 5.5 minutes after treatment with E4031, respectively.

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Figure 23 shows extra-cellular field potentials recorded for mouse stem-cell derived cardiomyocytes obtained using a device of the present invention, where the recording electrode is a circle-on-line electrode structure and the reference electrode is a gold wire electrode that is introduced into the well after cell seeding. **Figure 23A, 23B, 23C, 23 D and 23E** show the recorded waveforms before treatment, 10 seconds, 50 seconds, 3 minutes and 9 minutes after treatment with 3 uM Quinidine, respectively.

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Figure 24 shows parallel impedance monitoring of beating of mouse stem-cell derived cardiomyocytes obtained using a device of the present invention, where the impedance is monitored using an electrode array comprising two electrode structures each of which comprises multiple circle-on-line electrode elements. **Figure 24A, 24B, 24C, and 24 D** show the recorded impedance beating signals before treatment, 1.5 minute, ~ 3 minute and ~ 11 minute after treatment with 3 uM Quinidine, respectively.

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Figure 25 shows extra-cellular field potentials recorded for primary cardiomyocytes obtained using a device of the present invention, where the recording electrode is a circle-on-line electrode structure and the reference electrode is a gold wire electrode that is introduced into the well after cell seeding.

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Figure 26 shows a device of the present invention wherein the well (2605) of the device comprises a recording electrode (2601) for extra-cellular recording, an interdigitated electrode array (2602) comprising two electrode structures (2603 and 2606) each of which comprises multiple electrode elements. The electrodes are positioned on non-conductive substrate 2604. Extracellular recording of field potentials can be achieved by amplifying and recording voltage signals between the recording electrode 2601 and one electrode structure 2603 (or 2606). The impedance measurement can be conducted by monitoring impedance between electrode structures 2603 and 2606.

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Figure 27 shows the design of an electrode array used for combined cell-impedance measurement and extra-cellular recording.

5 **Figure 28** shows the impedance beating result obtained for the Cor. At cells (mouse ES derived cardiomyocytes) for ~ 12 seconds with time-solution about 28.8 ms per data point. The impedance data is represented with cell index, a dimension less parameter.

10 **Figure 29** shows the extracellular field potential signals obtained for the Cor. At cells (mouse ES derived cardiomyocytes) using the electrode array in **Figure 27** for three different recording modes, including: (1) ECR electrode versus impedance electrode-structure, (2) ECR electrode versus external, gold, reference electrode and (3) impedance electrode-structure versus external, gold, reference electrode.

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Figure 30 shows the design of another electrode array used for combined cell-impedance measurement and extra-cellular recording.

20 **Figure 31** shows the impedance beating result obtained for the Cor. At cells (mouse ES derived cardiomyocytes) for ~ 12 seconds with time-solution about 28.8 ms per data point. The impedance data is represented with cell index, a dimension less parameter.

25 **Figure 32** shows the extracellular field potential signals obtained for the Cor. At cells (mouse ES derived cardiomyocytes) using the electrode array in **Figure 20** for three different recording modes, including: (1) ECR electrode versus impedance electrode-structure, (2) ECR electrode versus external, gold, reference electrode and (3) impedance electrode-structure versus external, gold, reference electrode.

30 **Figure 33** shows top view and cross-sectional view of a device of the present invention.

Figure 34 shows a device of the present invention where the well of the device **3405** comprises a recording electrode **3401** and an interdigitated electrode array **3406** on the substrate **3404**, and an external, reference electrode **3402**.

5 DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow. Further, unless defined
10 otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set
15 forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

20 As used herein, “biocompatible membrane” means a membrane that does not have deleterious effects on cells, including the viability, attachment, spreading, motility, growth, cell division or cell beating.

A “biomolecular coating” or a “biological molecule coating” is a coating on a surface
25 that comprises a molecule that is a naturally occurring biological molecule or biochemical, or a biochemical derived from or based on one or more naturally occurring biomolecules or biochemicals. For example, a biological molecule coating can include an extracellular matrix component (e.g., fibronectin, collagens), or a derivative thereof, or can comprise a biochemical such as polylysine or polyornithine,
30 which are polymeric molecules based on the naturally occurring biochemicals lysine and ornithine. Polymeric molecules based on naturally occurring biochemicals such as amino acids can use isomers or enantiomers of the naturally-occurring biochemicals.

A “cell-free zone” is a region of the device where cells, when added to the device, are not physically present. The “cell-free zone” may be a region on the substrate, which is blocked from cell access. The “cell-free zone” may be a spatial position free from contact with the substrate such as a spatial position above a substrate base and within the volume of the well. The “cell-free zone” should permit the passage of electrical current such as through a conductive medium. For example, a reference electrode or external electrode may be suspended in a cell-free zone, free from contact with the substrate, yet remain electrically coupled to an extracellular recording electrode disposed on the substrate.

A “cell-free electrode” is an electrode that is free from contact with all cells present in the device.

The term “free from contact” or “free from direct contact” refers to two individuals, whether cells or apparatus components that lack contacting surfaces. A cell suspended in medium, in which only the medium contacts an electrode is “free from contact” or “free from direct contact” with the electrode.

An “organic compound coating” is a coating on a surface that includes an organic compound. For example an organic compound may include a natural ligand or an agonist or an antagonist for a cell surface receptor.

An “extracellular matrix component” is a molecule that occurs in the extracellular matrix of an animal. It can be a component of an extracellular matrix from any species and from any tissue type. Nonlimiting examples of extracellular matrix components include laminins, collagens fibronectins, other glycoproteins, peptides, glycosaminoglycans, proteoglycans, etc. Extracellular matrix components can also include growth factors.

An “electrode” is a structure having a high electrical conductivity, that is, an electrical conductivity much higher than the electrical conductivity of the surrounding materials, which in the present invention are typically nonconductive. An “extracellular recording electrode” or “recording electrode” or “ECR electrode” is such a structure used to detect electrical signal corresponding to extracellular field

potential of the cell or cell population. For instance, a “recording electrode” may be used to monitor the extracellular field potential of a cardiomyocyte during the generation of membrane action potentials. A “reference electrode” is the complementary structure used to complete the electrical circuit during extracellular recording. An “impedance electrode” or an “impedance measurement electrode” or “impedance measurement electrode structure” is a structure, such as an electrode, used for impedance monitoring. An “impedance electrode” may also operate as an extracellular recording electrode and thus may provide both impedance monitoring and extracellular recording measurements, albeit at different time points.

As used herein, an “electrode structure” refers to a single electrode, particularly one with a complex structure (as, for example, a spiral electrode structure), or a collection of at least two electrode elements that are electrically connected together. All the electrode elements within an “electrode structure” are electrically connected.

As used herein, “electrode element” refers to a single structural feature of an electrode structure, such as, for example, a fingerlike or branched projection of an interdigitated electrode structure. An electrode structure may have a plurality of electrode elements.

As used herein, a “unitary electrode structure” refers to a single electrode that is unbranched. That is, a “unitary electrode structure” does not include a plurality of electrode elements. For example, an unitary electrode structure may be of a circle, a square or other geometry.

As used herein, an “electrode array” or “electrode structure unit” is two or more electrode structures that are constructed to have dimensions and spacing such that they can, when connected to a signal source, operate as a unit to generate an electrical field in the region of spaces around the electrode structures. Preferred electrode structure units of the present invention can measure impedance changes due to cell attachment to an electrode surface. Non-limiting examples of electrode structure units are interdigitated electrode structure units and concentric electrode structure units.

An “electrode bus” is a portion of an electrode that connects individual electrode elements or substructures. An electrode bus provides a common conduction path from

individual electrode elements or individual electrode substructures to another electrical connection. In the devices of the present invention, an electrode bus can contact each electrode element of an electrode structure and provide an electrical connection path to electrical traces that lead to a connection pad.

5

“Electrode traces” or “electrically conductive traces” or “electrical traces”, are electrically conductive paths that extend from electrodes or electrode elements or electrode structures toward one end or boundary of a device or apparatus for connecting the electrodes or electrode elements or electrode structures to an analyzer or amplifier, such as an impedance amplifier, a voltage amplifier and the like. Both
10 impedance electrodes and extracellular recording electrodes may connect to an “electrode trace.” The end or boundary of a device may correspond to the connection pads on the device or apparatus.

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A “connection pad” is an area on an apparatus or a device of the present invention which is electrically connected to at least one electrode or all electrode elements within at least one electrode structure on an apparatus or a device and which can be operatively connected to external electrical circuits (e.g., an impedance measurement circuit or a signal source or an extracellular voltage signal amplifier). The electrical
20 connection between a connection pad and an impedance measurement circuit, an extracellular recording circuit or a signal source can be direct or indirect, through any appropriate electrical conduction means such as leads or wires. Such electrical conduction means may also go through electrode or electrical conduction paths located on other regions of the apparatus or device.

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“Interdigitated” means having projections coming one direction that interlace with projections coming from a different direction in the manner of the fingers of folded hands (with the caveat that interdigitated electrode elements preferably do not contact one another).

30

As used herein, a “high probability of contacting an electrode element” means that, if a cell is randomly positioned within the sensor area of a device or apparatus of the present invention, the probability of a cell (or particle) contacting an electrode element, calculated from the average diameter of a cell used on or in a device or

apparatus of the present invention, the sizes of the electrode elements, and the size of the gaps between electrode elements, is greater than about 50%, more preferably greater than about 60%, yet more preferably greater than about 70%, and even more preferably greater than about 80%, greater than about 90%, or greater than about 95%.

5

As used herein, “at least two electrodes fabricated on the substrate” means that the at least two electrodes are fabricated or made or produced on the substrate. The at least two electrodes can be on the same side of the substrate or on the different side of the substrate. The substrate may have multiple layers, the at least two electrodes can be either on the same or on the different layers of the substrate.

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As used herein, “at least two electrodes fabricated to a same plane of the substrate” means that, if the nonconducting substrate has multiple layers, the at least two electrodes are fabricated to the same layer of the substrate.

15

As used herein, “an electrode positioned on a different plane” refers to the positioning of an electrode, typically an external electrode or reference electrode, above, below or along a different surface angle than that which it is compared. An “electrode positioned on a different plane” may be parallel to that of the first.

20

As used herein, “the . . . electrodes (or electrode structures) have substantially the same surface area” means that the surface areas of the electrodes referred to are not substantially different from each other, so that the impedance change due to cell attachment or growth on any one of the electrodes (or electrode structures) referred to will contribute to the overall detectable change in impedance to a same or similar degree as the impedance change due to cell attachment or growth on any other of the electrodes (or electrode structures) referred to. In other words, where electrodes (or electrode structures) have substantially the same surface area, any one of the electrodes can contribute to overall change in impedance upon cell attachment or growth on the electrode. In most cases, the ratio of surface area between the largest electrode and the smallest electrode that have “substantially the same surface area” is less than 10. Preferably, the ratio of surface area between the largest electrode and the smallest electrode of an electrode array is less than 5, 4, 3, 2, 1.5, 1.2 or 1.1. More

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preferably, the at least two electrodes of an electrode structure have nearly identical or identical surface area.

As used herein, “the device has a surface suitable for cell attachment or growth”

- 5 means that the electrode and/or non-electrode area of the apparatus has appropriate physical, chemical or biological properties such that cells of interest can viably attach on the surface and new cells can continue to attach, while the cell culture grows, on the surface of the apparatus. However, it is not necessary that the device, or the surface thereof, contain substances necessary for cell viability or growth. These
- 10 necessary substances, e.g., nutrients or growth factors, can be supplied in a medium. Preferably, when a suspension of viable cardiomyocytes, neuron cells, muscle cells or other excitable cells or other adherent cells such as epithelial cells or endothelial cells is added to the “surface suitable for cell attachment” when at least 50% of the cells are adhering to the surface within twelve hours. More preferably, a surface that is
- 15 suitable for cell attachment has surface properties so that at least 70% of the cells are adhering to the surface within twelve hours of plating (i.e., adding cells to the chamber or well that comprises the said device). Even more preferably, the surface properties of a surface that is suitable for cell attachment results in at least 90% of the cells adhering to the surface within twelve hours of plating. Most preferably, the
- 20 surface properties of a surface that is suitable for cell attachment results in at least 90% of the cells adhering to the surface within eight, six, four, two hours of plating.

As used herein, “detectable change in impedance between or among said electrodes” (or “detectable change in impedance between or among the electrode structures”)

- 25 means that the impedance between or among the electrodes (or electrode structures) would have a significant change that can be detected by an impedance analyzer or impedance measurement circuit when cells attach on the electrode surfaces. The impedance change refers to the difference in impedance values when cells are attached to the electrode surface and when cells are not attached to the electrode
- 30 surface, or when the number, type, activity, adhesiveness, or morphology of cells attached to the electrode-comprising surface of the apparatus changes. In most cases, the change in impedance is larger than 0.1% to be detectable. Preferably, the detectable change in impedance is larger than 1%, 2%, 5%, or 8%. More preferably, the detectable change in impedance is larger than 10%. Impedance between or among

electrodes is typically a function of the frequency of the applied electric field for measurement. “Detectable change in impedance between or among the electrodes” does not require the impedance change at all frequencies being detectable.

“Detectable change in impedance between or among said electrodes” only requires a detectable change in impedance at any single frequency (or multiple frequencies). In addition, impedance has two components, resistance and reactance (reactance can be divided into two categories, capacitive reactance and inductive reactance).

“Detectable change in impedance between or among said electrodes” requires only that either one of resistance and reactance has a detectable change at any single frequency or multiple frequencies. In the present application, impedance is the electrical or electronic impedance. The method for the measurement of such impedance is achieved by, (1) applying a voltage between or among the electrodes at a given frequency (or multiple frequencies, or having specific voltage waveform) and monitoring the electrical current through said electrodes at the frequency (or multiple frequencies, or having specific waveform), dividing the voltage amplitude value by the current amplitude value to derive the impedance value; (2) applying an electric current of a single frequency component (or multiple frequencies or having specific current wave form) through said electrodes and monitoring the voltage resulted between or among said electrodes at the frequency (or multiple frequencies, or having specific waveform), dividing the voltage amplitude value by the current amplitude value to derive the impedance value; (3) other methods that can measure or determine electric impedance. Note that in the description above of “dividing the voltage amplitude value by the current amplitude value to derive the impedance value”, the “division” is done for the values of current amplitude and voltage amplitude at same frequencies. Measurement of such electric impedance is an electronic or electrical process that does not involve the use of any reagents.

As used herein, “multiple pairs of electrodes or electrode structures spatially arranged according to wells of a multi-well microplate” means that the multiple pairs of electrodes or electrode structures of a device or apparatus are spatially arranged to match the spatial configuration of wells of a multi-well microplate so that, when desirable, the device can be inserted into, joined with, or attached to a multiwell plate (for example, a bottomless multiwell plate) such that multiple wells of the multi-well microplate will comprise electrodes or electrode structures.

As used herein, “arranged in a row-column configuration” means that, in terms of electric connection, the position of an electrode, an electrode array or a switching circuit is identified by both a row position number and a column position number.

5

As used herein, “each well contains substantially same number . . . of cells” means that the lowest number of cells in a well is at least 50% of the highest number of cells in a well. Preferably, the lowest number of cells in a well is at least 60%, 70%, 80%, 90%, 95% or 99% of the highest number of cells in a well. More preferably, each

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As used herein, “each well contains . . . same type of cells” means that, for the intended purpose, each well contains same type of cells; it is not necessary that each well contains exactly identical type of cells. For example, if the intended purpose is

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that each well contains mammalian cells, it is permissible if each well contains same type of mammalian cells, *e.g.*, human cells, or different mammalian cells, *e.g.*, human cells as well as other non-human mammalian cells such as mice, goat or monkey cells, etc.

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As used herein, “each well contains . . . serially different concentration of a test compound” means that each well contains a test compound with a serially diluted concentrations, *e.g.*, an one-tenth serially diluted concentrations of 1 M, 0.1 M, 0.01 M, etc.

25

As used herein, “dose-response curve” means the dependent relationship of response of cells on the dose concentration of a test compound. The response of cells can be measured by many different parameters. For example, a test compound is suspected to have cytotoxicity and cause cell death. Then the response of cells can be measured by percentage of non-viable (or viable) cells after the cells are treated by the test

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compound. Plotting this percentage of non-viable (or viable) cells as a function of the dose concentration of the test compound constructs a dose response curve. In the present application, the percentage of non-viable (or viable) cells can be expressed in terms of measured impedance values, or in terms of cell index derived from impedance measurement, or in terms of cell change indexes. For example, for a give

cell type and under specific cellular physiological condition (e.g., a particular cell culture medium), cell index can be shown to have a linear correlation or positive correlation with the number of viable cells in a well from which cell index was derived from the impedance measurement. Thus, in the present application, one can
5 plot cell index as a function of the dose concentration of the test compound to construct a “dose-response curve”. Note that, generally, cell index not only correlate with the number of viable cells in the wells but also relate to the cell morphology and cell attachment. Thus plotting cell index versus dose concentration provides information not only about number of cells but also about their physiological status
10 (e.g. cell morphology and cell adhesion). Furthermore, an important advantage offered by the system and devices of the present invention is that in a single experiment, one can obtain “dose-response curves” at multiple time points since the system allows for the continuous monitoring of cells and provides impedance measurement at many time points over a time range as short as a few minutes to as
15 long as days or weeks.

As used herein, “microelectrode strip or electrode strip” means that a non-conducting substrate strip on which electrodes or electrode structure units are fabricated or incorporated. The non-limiting examples of the non-conducting substrate strips
20 include polymer membrane, glass, plastic sheets, ceramics, insulator-on-semiconductor, fiber glass (like those for manufacturing printed-circuits-board). Electrode structure units having different geometries can be fabricated or made on the substrate strip by any suitable microfabrication, micromachining, or other methods. Non-limiting examples of electrode geometries include interdigitated electrodes,
25 circle-on-line electrodes, diamond-on-line electrodes, castellated electrodes, spiral electrodes or sinusoidal electrodes. Characteristic dimensions of these electrode geometries may vary from as small as less than 5 micron, or less than 10 micron, to as large as over 200 micron, over 500 micron, over 1 mm. The characteristic dimensions of the electrode geometries refer to the smallest width of the electrode elements, or
30 smallest gaps between the adjacent electrode elements, or size of a repeating feature on the electrode geometries. The microelectrode strip can be of any geometry for the present invention. One exemplary geometry for the microelectrode strips is rectangular shape – having the width of the strip between less than 50 micron to over 10 mm, and having the length of the strip between less than 60 micron to over 15 mm.

An exemplary geometry of the microelectrode strips may have a geometry having a width of 200 micron and a length of 20 mm. A single microelectrode strip may have two electrodes serving as a measurement unit, or multiple such two-electrodes serving as multiple measurement units, or a single electrode structure unit as a measurement unit, or multiple electrode structure units serving as multiple electrode structure units. In one exemplary embodiment, when multiple electrode structure units are fabricated on a single microelectrode strip, these electrode structure units are positioned along the length direction of the strip. The electrode structure units may be of squared-shape, or rectangular-shape, or circle shapes. Each of electrode structure units may occupy size from less than 50 micron by 50 micron, to larger than 2 mm x 2mm.

As used herein, “sample” refers to anything which may contain a moiety to be isolated, manipulated, measured, quantified, detected or analyzed using apparatuses, microplates or methods in the present application. The sample may be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include suspension of cells in a medium such as cell culture medium, urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s). The biological samples may further include cell suspensions, solutions containing biological molecules (e.g. proteins, enzymes, nucleic acids, carbohydrates, chemical molecules binding to biological molecules) .

As used herein, a “liquid (fluid) sample” refers to a sample that naturally exists as a liquid or fluid, *e.g.*, a biological fluid. A “liquid sample” also refers to a sample that naturally exists in a non-liquid status, *e.g.*, solid or gas, but is prepared as a liquid, fluid, solution or suspension containing the solid or gas sample material. For example, a liquid sample can encompass a liquid, fluid, solution or suspension containing a biological tissue.

A "compound" or "test compound" is any compound whose activity or direct or indirect effect or effects on cells is investigated in any assay. A test compound can be any compound, including, but not limited to, a small molecule, a large molecule, a molecular complex, an organic molecule, an inorganic molecule, a biomolecule or biological molecule such as but not limited to a lipid, a steroid, a carbohydrate, a fatty acid, an amino acid, a peptide, a protein, a nucleic acid, or any combination thereof. A test compound can be a synthetic compound, a naturally occurring compound, a derivative of a naturally-occurring compound, etc. The structure of a test compound can be known or unknown. In one application of the present invention, a compound is capable of, or is suspected of, effecting cell adhesion or cell spreading. In another application of present invention, a compound is capable of, or is suspected of, stimulating or inhibiting cell adhesion or cell spreading. In still another application, a compound is capable of, or is suspected of, interacting with cells (for example, binding to cell surface receptor, or inhibiting certain intracellular signal transduction pathway, or activating cells).

A "known compound" is a compound for which at least one activity is known. In the present invention, a known compound preferably is a compound for which one or more direct or indirect effects on cells is known. Preferably, the structure of a known compound is known, but this need not be the case. Preferably, the mechanism of action of a known compound on cells is known, for example, the effect or effects of a known compound on cells can be, as nonlimiting examples, effects on cell beating, cell viability, cell adhesion, apoptosis, cell differentiation, cell proliferation, cell morphology, cell cycle, IgE-mediated cell activation or stimulation, receptor-ligand binding, cell number, cell quality, cell cycling, cell adhesion, cell spreading, etc.

An "impedance value" is the impedance measured for electrodes in a well with or without cells present. Impedance is generally a function of the frequency, i.e., impedance values depend on frequencies at which the measurement was conducted. For the present application, impedance value refers to impedance measured at either single frequency or multiple frequencies. Furthermore, impedance has two components, one resistance component and one reactance component. Impedance value in the present application refers to resistance component, or reactance component, or both resistance and reactance component. Thus, when "impedance

value” was measured or monitored, we are referring to that, resistance, or reactance, or both resistance and reactance were measured or monitored. In many embodiments of the methods of the present application, impedance values also refer to parameter values that are derived from raw, measured impedance data. For example, cell index, or normalized cell index, or delta cell index could be used to represent impedance values.

A "Cell Index" or "CI" is a parameter that can be derived from measured impedance values and that can be used to reflect the change in impedance values. There are a number of methods to derive or calculate Cell Index. The details of the method for calculating Cell Index, Normalized Cell Index, Delta Cell Index and cell change index can be found in United States patent application No. 10/705,447, filed on November 10, 2003; United States patent application No. 10/705,615, filed on November 10, 2003; United States patent application No. 10/987,73, filed on Nov 12, 2004; United States patent application No. 11/055,639, filed on Feb 9 2005; United States patent application No. 11/198,831, filed on Aug 4, 2005; United States patent application No. 11/197,994, filed on Aug 4, 2005; United States patent application No. 11/235,938, filed on Sept 27, 2005, all of them are incorporated here by reference.

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. Generally, for an assay involving treatment of the cells with compounds or with other bio-manipulation of the cells, the reference time point is the last time point for impedance measurement before the treatment of the cells.

A “delta cell index” at a given time point is calculated by subtracting the cell index at a standard time point from the cell index at the given time point. Thus, the delta cell index is the absolute change in the cell index from an initial time (the standard time point) to the measurement time.

A "Cell Change Index" or "CCI" is a parameter derived from Cell Index and “CCI” at a time point is equal to the 1st order derivative of the Cell Index with respect to time, divided by the Cell Index at the time point. In other words, CCI is calculated as

$$CCI(t) = \frac{dCI(t)}{CI(t) \cdot dt}.$$

5 The term “extracellular recording” refers to measuring, monitoring and/or recording of electric potential difference between two electrodes typically caused by ionic movement or ionic current through the media or solution due to charge fluctuations across ion channels in a cell or in a group of cells. The cells are in the media or the solution. In contrast to intracellular recording where the recording electrodes are placed inside a cell through the cell membrane, the extracellular recording electrodes
10 are located outside of the cells.

B. Method to assess and quantify cardiomyocyte viability in vitro using cell sensor impedance technology

15 The methods and devices of the present invention may assess or quantify the viability of cardiomyocyte or cardiomyocyte precursor cells in addition to performing extracellular recording assays. Such assessment may be performed prior to performing an extracellular recording assay to assess the initial viability or characteristics of cells or may be performed periodically throughout the extracellular
20 recording process to continually monitor the viability or characteristics of cells, or may be performed in parallel with extracellular recording.

Isolated primary cardiomyocytes, ES-derived cardiomyocytes and adult stem cell-derived cardiomyocytes can be maintained in culture. These cells provide an excellent
25 model system to study the effect of drugs and other factors on cardiomyocyte viability.

Exemplary steps involved in using an impedance-monitoring system for measurement of cardiomyocyte viability include:

- 30 (1) Provide a single-well or multi-well device that comprise microelectrode arrays in well(s) of the device, which can be used for monitoring cell-substrate impedance.

- (2) Optionally coat wells of the device with either fibronectin or other matrix proteins.
- (3) Seed either embryonic stem cells (ES cells) of mammalian origin, mammalian adult stem cell-derived cardiomyocytes or primary cardiomyocytes isolated directly from mammalian heart tissue at specific seeding densities to the wells of the device.
- (4) Allow the cells to attach and spread.
- (5) Monitor cardiomyocyte viability over time using the impedance-monitoring system to monitor electrode impedance at pre-specified intervals of time (non-limiting examples of the time include 5, 15, 30 minutes, 1hr and 2 hrs) for specified length of time such as 12, 24, 48, 72 hours or longer.

One example of the impedance measurement system is ACEA Biosciences' RT-CES system, where the device is ACEA E-PLATE in the form of microtiter plates whose wells comprise microelectrode structures. Thus in a related embodiment, exemplary steps involved in using the RT-CES system for measurement of cardiomyocyte viability include:

- (1) Optionally coat E-PLATES with either fibronectin or other matrix proteins.
- (2) Seed either embryonic stem cells (ES cells) of mammalian origin, mammalian adult stem cell-derived cardiomyocytes or primary cardiomyocytes isolated directly from mammalian heart tissue at specific seeding densities to the wells of the device.
- (3) Allow the cells to attach and spread.
- (4) Monitor cardiomyocyte viability over time using the impedance-monitoring system to monitor electrode impedance at pre-specified intervals of time (non-limiting examples of the time include 5, 15, 30 minutes, 1hr and 2 hrs) for specified length of time such as 12, 24, 35, 48, 72 hours or longer.

As an example, we describe here the use of the ACEA RT-CES system to measure and monitor the attachment, growth and viability of mouse ES-derived cardiomyocytes which were seeded at different seeding densities. Mouse ES cells were seeded at a density ranging from 3000 cells to 50,000 cells per well in ACEA E-PLATES that had been precoated with fibronectin. The attachment, growth and viability of the cells were monitored on the RT-CES system measuring impedance signal in the form of cell index every 30 minutes for 48 hours. At about 48 hrs after cell seeding, the growth of the cells had ceased and the appearance of beating cardiomyocytes were evident as judged by looking at the cells inside the E-PLATE under the microscope. As such, these cells would be suitable for use, for example, in extracellular recording experiments to determine the effect of external stimuli, such as changes in cardiomyocyte beating after administration of a test compound, or in the experiments to monitor cardiomyocyte beating through impedance measurement at milli-second time resolution. **Figure 1** shows the cell index curves measured on RT-CES system for 4 different seeding densities (3750, 7500, 15,000 and 30,000 cells per well) of mouse ES-derived cardiomyocytes. For such long term measurement, cell electrode impedance and corresponding cell indices were measured at about 15 minute intervals. Based on the cell index growth and viability curves, it is evident that the extent of the impedance signal correlates well with the seeding density of viable ES-derived cardiomyocytes.

C. Method to assess and quantify modulation of cardiomyocyte viability *in vitro* using cell sensor impedance technology

Certain cardiotoxic drugs can directly affect the viability of cardiomyocytes. Accordingly, continuing to monitor the viability of cardiomyocytes over time may be used to supplement data obtained from extracellular recording of cells. Exemplary steps involved in using an impedance-measurement system for measurement of loss of viability of cardiomyocyte include:

- (1) Optionally coat E-PLATES with either fibronectin or other matrix proteins.
- (2) Seed either embryonic stem cells (ES cells) of mammalian origin, mammalian adult stem cell-derived cardiomyocytes or primary

cardiomyocytes isolated directly from mammalian heart tissue at specific seeding densities to the wells of the device.

(3) Allow the cells to attach and spread.

(4) Monitor cardiomyocyte viability over time using the impedance-monitoring system to monitor electrode impedance at pre-specified intervals of time for specified length of time such as 12, 24, 37, 48, 72 hours or longer

(5) At certain time after cell seeding, treat the cell with cytotoxic agent at one or more concentration; using the vehicle that the agent is dissolved in as a control.

(6) Continue monitoring the cardiomyocytes at pre-specified intervals of time for specified length of time such as additional 12, 24, 48, 72 hours or longer

(7) Quantify the extent of cardiotoxicity by normalizing the cell index values immediately prior to agent addition and determine the normalized cell index at a given time point after agent addition; alternatively the rate of cytotoxicity can also be quantified for a given time period after compound addition for a given agent concentration or a group of concentrations. The extent of cytotoxicity can be expressed as IC-50 value which quantifies the activity of the agent with respect to the cardiomyocytes.

As an example, we describe here the use of the ACEA RT-CES system to measure and monitor the attachment and growth of mouse ES cells derived cardiomyocytes and subsequently treated with a cytotoxic agent (**Figure 2**). Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells per well in ACEA E-Plates that had been precoated with fibronectin. The attachment and growth of the cells were monitored on RT-CES system for 72 hours and then treated with increasing doses of the compound of sodium dichromate dehydrate which is known to induce cytotoxicity. According to **Figure 2A** of the plot of normalized cell index for cells in different wells treated with different concentrations of sodium dichromate dehydrate (SDD), SDD causes a concentration dependent decrease in viability of ES-derived cardiomyocytes. To quantify the extent of sodium dichromate dehydrate activity against the cardiomyocytes, the normalized cell indices at 24 hrs after compound

treatment were plotted against the log of the corresponding sodium dichromate dehydrate concentrations. From the sigmoidal curve, shown in **Figure 2B**, half maximal activity or IC-50 value of 1.48 uM was derived for the compound.

5 D. Method to assess and quantify modulation of cardiomyocyte morphology *in vitro* using cell sensor impedance technology

Certain cardiotoxic drugs can illicit their effect by affecting the morphological aspects of cardiomyocyte morphology. For example, it is well known that compounds such as
10 b-2 adrenergic receptor agonists can induce morphological changes resulting in an elongated cardiomyocyte morphology, otherwise known as hypertrophy.

Morphological changes can occur immediately in the order of minutes as with certain GPCR agonists or can be of longer duration detectable over several days. The time resolution of the RT-CES system can be used to distinguish between different kinds
15 of morphological effects. The steps involved in using an impedance-monitoring system on the RT-CES system for measurement of morphological modulation of cardiomyocytes include:

(1) Optionally coat E-PLATES with either fibronectin or other matrix proteins.

20 (2) Seed either embryonic stem cells (ES cells) of mammalian origin, mammalian adult stem cell-derived cardiomyocytes or primary cardiomyocytes isolated directly from mammalian heart tissue at specific seeding densities to the wells of the device.

(3) Allow the cells to attach and spread.

25 (4) Monitor cardiomyocyte viability over time using the impedance-monitoring system at prespecified intervals of time for 12, 24, 48, 72 hours or longer

(5) At certain time after cell seeding, treat the cell with agents that may cause morphology changes at one or more concentration; using the
30 vehicle that the agent is dissolved in as a control.

(6) Continue monitoring the cardiomyocytes at 1 minute intervals for at least 1-2 hours to capture any immediate morphological changes and continue to monitor at 30 minutes intervals of time for additional 12, 24, 48, 72 hours or longer to detect long term morphological changes

(7) Quantify the extent of morphological change by normalizing the cell index values immediately prior to agent addition and determine the normalized cell index at a given time point after agent addition; The extent of morphological change can be expressed as EC-50 value which quantifies the activity of the agent with respect to the cardiomyocyte shape changes.

As an example, we describe here the use of the ACEA RT-CES system to measure and monitor the attachment and growth of mouse ES cells derived cardiomyocytes and subsequently treated with isoproteranol, a β_2 adrenergic receptor agonist known to induce hypertrophy (**Figure 3A**). Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells per well in ACEA E-PLATES that had been precoated with fibronectin. The attachment and growth of the cells were monitored on RT-CES system for 72 hours and then treated with increasing doses of the compound isoproteranol. According to **Figure 3A**, isoproteranol causes a concentration dependent change in cell index readings. The timing of the cell index change is consistent with a change in the morphology of the cells which we have shown previously for other GPCR agonists in primary cells (Yu et al (2006) Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors; Analytical Chemistry, Vol 78, pages 35-43). To quantify the extent of isoproteranol-induced morphological changes in mouse ES-derived cardiomyocytes, the normalized cell indices were plotted against the log of the corresponding isoproteranol concentrations (**Figure 3B**). From the sigmoidal curve generated a half maximal activity or IC-50 value of 3.1 nM was derived for the compound.

E. Method to assess and quantify cardiomyocyte beating *in vitro* using cell sensor impedance technology

Isolated primary cardiomyocytes as well as ES-derived cardiomyocytes retain the ability to beat in culture. These cells provide an excellent model system to study

cardiomyocyte function *in vitro*, especially with regards to cardiotoxicity. A number of cardiotoxic drugs are known to affect certain heart channels, such as the ERG channels, that are involved in excitation-contraction coupling of cardiomyocytes. Cardiomyocytes have an innate ability to undergo mechanotransduction, that is that the spontaneous force generation of the beating cardiomyocyte is translated to intracellular biochemical signals. Membrane receptors such as integrins, ion channels and other proteins have been shown to play a crucial role in cardiac mechanotransduction and lead to a continuous and rhythmic dynamics of the cardiac actin cytoskeleton and morphology. Because the impedance-based technology and system can sensitively and precisely detect transient changes in morphology and adhesive capacity of the cells, it can be used to monitor cardiomyocyte beating *in vitro*.

The steps involved in using an impedance-measurement system for measurement of cardiomyocyte beating include:

- (1) Provide a single-well or multi-well device that comprise microelectrode arrays in well(s) of the device, which can be used for monitoring cell-substrate impedance.
- (2) Optionally coat wells of the device with either fibronectin or other matrix proteins.
- (3) Seed either embryonic stem cells (ES cells) of mammalian origin or primary cardiomyocytes at specific seeding densities to the wells of the device.
- (4) Allow the cells to attach and spread.
- (5) After a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitor cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using milli-second kinetic readout to resolve the individual beat cycles of the cells.

The milli-second kinetic readout requires that the impedance measurement system can provide impedance measurement data at milli-second time resolution. In other words, the time difference between two consecutive impedance measurement for a well shall be in the range of milli-seconds (e.g., less than 500 milli-second, less than 300 milli-

second, less than 100 milli-second, less than 40 milli-second, less than 30 milli-second, less than 20 milli-second, less than 10 millisecond, or less than 1 millisecond or faster). The milli-second kinetic readout is required to resolve the individual beat cycles of the cells. Thus, the time resolution for the impedance measurement should
5 allow the system to perform measurement at least two time points for each beat cycle, or at more than two points for each beat cycle. These milli-second kinetic readouts may be performed within extracellular recording experiments by switching between an extracellular recording mode and impedance mode via a switching means. The fast kinetic readouts associated with such an impedance based system permits the user to
10 obtain multiple impedance measurements to obtain the impedance data for one or more beating cycle of the cardiomyocytes between extracellular recording measurements.

One example of the impedance measurement systems is an improved fast-impedance-measurement system from ACEA Biosciences, where the device is the E-PLATE in
15 the form of microtiter plates whose wells comprise microelectrode structures. An important aspect of the present invention is that the impedance measurement circuitry together electronic switching circuitry and the associated is capable of measuring electronic impedances of the one or more wells at milli-second time resolution, that is
20 to say, the time difference between two adjacent impedance measurements for each well is in the range of milli-seconds. This requirement is important, especially when the system comprises a device having multiple wells such as 4 wells, 8 wells, 16 wells or 96 wells. For example, if the system comprises a device having 96 wells, the system hardware and software should be capable of measuring the impedances of all
25 the 96 wells with milli-second time resolution between two adjacent impedance measurement points for each and every well. Preferably, the time difference between two adjacent measurement points for each and every given well is less than 500 milliseconds. More preferably, the time difference between two adjacent measurement points for each and every given well is less than 300 milliseconds.
30 More preferably, the time difference between two adjacent measurement points for each and every given well is less than 200 milliseconds. Still more preferably, the time difference between two adjacent measurement points for each and every given well is less than 100 milliseconds. Still more preferably, the time difference between two adjacent measurement points for each and every given well is less than 40

milliseconds. Still more preferably, the time difference between two adjacent measurement points for each and every given well is less than 20 milliseconds. Still more preferably, the time difference between two adjacent measurement points for each and every given well is less than 10 milliseconds.

5

A number of improvements in the impedance measurement circuitry, electronic switching circuitry, communication between impedance measurement circuitry and software can be used to achieve such milli-second time resolution. One aspect of improvements includes the use of fast processing electronic chips for analogue-to-digital conversion, for parallel digital signal processing and data calculation with
10 field-programmable gate array (FPGA) and for fast communication between the impedance measurement circuitry and software. Another aspect of improvements includes the use of multiple analogue-to-digital (AD) conversion channels so that analog electronic signals from multiple channels can be converted to digital signals
15 simultaneously. Such parallel AD conversion is important, particular for the system having multiple wells, each of which's measurement time resolution is required to be in the milli-second resolution. Another very important aspect of improvements is to replace typical working mode of "measurement of one-well's impedance at a time" with a mode of "measurement of multiple-wells' impedance at a time". In "one-well
20 at a time" mode, when the software issue a command for measuring one well's impedance, the measurement circuitry would perform the measurement for one well including signal generation to the well, converting the voltage signal and the electric current signal for the well to digital signal, digitally processing the signals to do impedance calculation and sending the well's impedance data to the computer over
25 the communication line between the impedance measurement circuitry and the computer. The system will not perform any measurement for another well until the completion of the measurement of this well and until receiving another command for the measurement of another well. In "multiple-wells at a time" mode, the software would issue a command for measuring multiple wells' impedance. The measurement
30 circuitry would simultaneously or nearly simultaneously perform signal conversion, signal processing and impedance calculation for multiple wells. The multiple impedance data for the multiple wells would be sent over the communication lines to the computer sequentially with one well's data at time or simultaneously with more than one well's data being sent at a time. In this "measurement of multiple-wells'

impedance at a time” mode, the system may be performing multiple tasks simultaneously, for example, while one well’s impedance data is being measured and calculated, another well’s impedance data may be communicated and sent over the communication lines to the computer.

5

The steps involved in using the system for measurement of cardiomyocyte function may include:

- (1) Optionally coat E-PLATES with either fibronectin or other matrix proteins.
- 10 (2) Seed either ES cells of mammalian origin or primary cardiomyocytes at specific seeding densities.
- (3) Allow the cells to attach and spread.
- (4) After a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitor cardiomyocyte
- 15 beating using the RT-CES system by using milli-second kinetic readout to resolve the individual beat cycles of the cells.

As an example, we describe here the use of the ACEA RT-CES system to measure and monitor the beating of cardiomyocytes using fast kinetic software. Mouse ES cells

20 were seeded at a density of between 3,000 to 50,000 cells per well in ACEA E-PLATES that had been precoated with fibronectin. The attachment and growth of the cells were monitored on RT-CES system. **Figure 4** shows the cell index curves measured on RT-CES system for 4 individual wells, with cell culture medium as background starting from cell seeding to about 86 hrs after cell seeding. For such

25 long term measurement, cell electrode impedance and corresponding cell indices were measured at about 15 minute intervals. As evidenced on these plots, the cell index curves were rather smooth upto about 44-48 hrs, after which there were “noises” or “small-spikes” on the cell index curves. Such spikes were most evident after about 60 hrs. Such spikes in the impedance or cell index readout are associated with the

30 beating of the cells. During the synchronized beating of the cells, the cell morphology and cell adhesion/attachment to the electrodes change regularly in synchrony with the cell beating. Such regular or periodic changes in cell morphology and cell adhesion/attachment are then reflected in the changes in cell-electrode or cell-substrate impedances.

In order to monitor beating of cardiomyocytes, a specially designed software and measurement circuit hardware that are capable of millisecond impedance data acquisition (e.g., typically with time resolution between consecutive impedance measurement for a same well being less than 40 milli-seconds) was used to monitor the quick rhythmic beating of the cardiomyocytes. For such measurement, the background electrode impedance is measured with the cells inside the wells (note, this is in contrast with **Figures 1** and **4**, where the background measurement is performed using cell culture media). The software, together with specially designed hardware circuits was used to measure cardiomyocyte beating at distinct stages throughout its attachment and growth phases (**Figures 5**). For a baseline reference, the impedance measurement was done on the ES cells at 24 hours where the cells had not fully spread and formed a tight monolayer and even though the cells appear to beat when visualized under a microscope, they do so asynchronously and as a result no net beating signal is detected (**Figure 5A**). For plot, impedance readout has been converted into dimensionless cell indices. **Figure 5B** and **5C** shows that ES cells that had fully spread and formed tight junctions with neighboring cells at 48 hours and 72 hours respectively, show regular impedance-spikes which correlate with the beating frequency of cardiomyocytes as judged by microscopic observation .

To use the measured cell index curves, it is important to further derive various physiologically relevant parameters. Several important parameters may include, the beating rate of the cardiomyocytes (i.e., how many times the cells beat within a unit of time for example, a minute), the beating amplitude (i.e. the magnitude of the beating of cells in terms of impedance change) the average amplitude intensity in a unit time as well as the average length of time between the beats, time of rise for a beat, time of decay for a beat. Because of the unique and complex nature of the impedance readout signals (smaller signal amplitude, sampling time resolution may be limited by the hardware and the software used), appropriate methods or techniques are required for analyzing cell index curves to derive the above mentioned parameters.

For deriving the beating rates of cardiomyocytes, one method may be by counting how many peaks there are within a given time frame (for example, one minute). For this approach to work, the sampling time resolution has to be sufficiently high so that

the each beat of the cells does show a peak on the recorded cell index curves. In addition, determining a peak “automatically” also require some algorithm. For example, each peak would have to have one “rise” in cell index and also one “decay” in cell decay. Each “rise”-and-“decay” pair forms a single peak. The algorithm needs
5 to determine such “rise” and “decay” portions of the curves and then counts a peak.

Another method to derive or count the beating rates of cradiomyocytes is to perform a detailed signal analysis to derive the frequency components of the cell index curves and to derive the magnitude of each frequency components. One method of signal
10 analysis is Fourier transform of the cell index curve (of the time domain). Like above method, the sampling time-resolution needs to be sufficiently high so that each beat of the cells has at least three time points being measured. After performing Fourier transform, we would look for the frequency components having the largest magnitude and such frequency would correspond to, or be very close to, the beating frequency.

15 In addition, for such analysis, giving a fixed sampling time resolution, the more time points sampled for analysis, the more accurate it is for the analyzed beating frequency. **Figure 6A** and **Figure 6B** shows a pair of cell index curves and the corresponding beat rate based the above described Fourier transform. In **Figure 6A**, the cell index curves last from time zero to time 42 seconds. In **Figure 6B**, the
20 beating frequency for the traces in **Figure 6A** is analyzed using the method described here, i.e., Fourier transform followed by picking up the highest-magnitude frequency component. For each derived frequency data at one time moment in **Figure 6B**, cell index data from multiple time points (starting from previous 98 time points plus the time moment of interest) is used for analysis. Thus, the time axis in **Figure 6B** starts
25 from about 20 seconds to 42 seconds.

Another method deriving the beating rates of cardiomyocytes is to first determine the time length (ΔT in seconds) between two consecutive peaks and then calculate the beating rate according to the formula of “beats per minute = $60 / \Delta T$ ”. Thus for each
30 two-consecutive peaks, one can calculate one beating rate. Furthermore, one can plot this beating rate as a function of the time (of the first of the two consecutive peaks) to obtain the time dependency of the beating rates. **Figure 7A** shows an example of the time-dependent beat rates derived using this method, for mouse ES-derived cardiomyocytes treated with compound sotalol at a concentration of 4.4 μM .

Corresponding cell index data is shown in **Figures 7C and 7D**, where the time resolution between two adjacent points is 40 milli-seconds. In other words, a second in **Figures 7C and 7D** is equivalent to 40-milli-second.

- 5 For deriving the amplitude of the beating of the cardiomyocytes, there may also be different methods. One method is to analyze each peak and finding the peak maximum and the peak minimum. The amplitude is calculated by subtracting peak maximum by the peak minimum. Then, one can plot the peak amplitude as a function of the time of the peak to obtain the time dependency of the peak amplitude. **Figure**
10 **7B** shows an example of the time-dependent peak amplitudes derived using this method, for mouse ES-derived cardiomyocytes treated with compound sotalol at a concentration of 4.4 uM. Another approach may also be to use Fourier transform described above. Then based on derived Fourier coefficients, one can re-simulate time domain cell index curves and look for the peak magnitude from the simulated
15 curves

- For deriving the averaged length of time between the beats, there may also be different methods. For each identified peak, one can first determine a starting point of the peak. Then the time difference between two consecutive peaks at the two
20 starting points of the peak can be used for the length of time between the beats.

With the method of determining each peak, one can also calculate the time-of-rise of the peak and the time-decay-of the peak.

- 25 F. Method to assess the effect of known pharmacological agents, proteins, peptides, antibodies which modulate cardiac beating using the impedance-measurement system

- The method described in section E above offers a convenient label-free, real-time and
30 quantitative method for screening of modulators of cardiac function using a fast impedance-measurement system capable of milli-second measurement time resolution. The steps involved in screening for modulators of cardiomyocyte beating using an impedance-measurement system *in vitro* include:

- (1) Provide a single-well or multi-well device that comprise microelectrode arrays in well(s) of the device, which can be used for monitoring cell-substrate impedance.
- (2) Optionally coat wells of the device with either fibronectin or other matrix proteins.
- (3) Seed either embryonic stem cells (ES cells) of mammalian origin, mammalian adult stem cell-derived cardiomyocytes or primary cardiomyocytes at specific seeding densities to the wells of the device.
- (4) Allow the cells to attach and spread.
- (5) After a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitor cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using milli-second kinetic readout to resolve the individual beat cycles of the cells. This step should be done immediately prior to addition of pharmacological agent, in order to obtain a baseline of the cardiomyocyte beating frequency using fast measurement software and hardware to ensure milli-second kinetic readout signals.
- (6) Addition of the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency.

Similar to Section E, the milli-second kinetic readout requires that the impedance measurement system can provide impedance measurement data at milli-second time resolution. In other words, the time difference between two consecutive impedance measurement for a well shall be in the range of milli-seconds (e.g., less than 500 milli-second, less than 300 milli-second, less than 100 milli-second, less than 10 milliseond, or less than 1 millisecond or faster). The milli-second kinetic readout is required to resolve the individual beat cycles of the cells. Thus, the time resolution for the impedance measurement should allow the system to perform measurement at least two time points for each beat cycle, or at more than two points for each beat cycle.

One example of the impedance measurement systems is a fast impedance-measurement system from ACEA Biosciences, where the device is ACEA E-PLATE in the form of microtiter plates whose wells comprise microelectrode structures. The steps involved in screening for modulators of cardiomyocyte beating using the fast

5 impedance-measurement system *in vitro* include:

(1) Seeding cardiomyocytes, in E-Plates exactly as described in steps 1-4 in section E above for using the system for measurement of cardiomyocytes beating.

10 (2) Prior to addition of pharmacological agent, obtaining a baseline of the cardiomyocyte beating frequency using the fast kinetic RT-CES hardware and software.

(3) Addition of the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency.

15 In order to demonstrate the utility of the millisecond kinetic measurements, we first used two pharmacological agents, one known to suppress the heart rate and consequently cardiomyocyte beating and the other known to increase the heart rate and consequently the rate of cardiomyocyte beating. As Mouse ES cells were seeded in FN-coated E-PLATES and monitored for about 72 hours when the cells

20 differentiated into beating cardiomyocytes, as described in Section E. A baseline of the cardiomyocyte was taken for approximately 40 seconds using the specially designed fast kinetic data acquisition hardware and software which is capable of millisecond data acquisition and display. An agonist of muscarinic receptors, carbachol, which is known to slow down the heart rate was added to one well at a

25 final concentration of 333 nM and cardiomyocyte beating was monitored for 10 minutes (**Figure 8A**). The data clearly shows that carbachol significantly slows down the rate of cardiomyocyte beating from ~ 80 beats/min prior to carbachol addition to ~ 60 beats/min after carbachol addition (**Table I**). Alternatively, addition of isoproterenol at a final concentration of 4.4 uM significantly increased the rate of

30 cardiomyocyte beating from ~ 65 beats/min to 115-135 beats/min (**Figure 8B** and **Table I**). These data clearly show that the readout system and the fast kinetic software are sufficiently robust and sensitive to detect these changes in rate of cardiomyocyte beating even at very low compound concentrations. Similar to the cell index plot shown in **Figures 7C** and **7D**, the time resolution between two adjacent points in

Figures 8A and 8B is 40 milli-seconds. In other words, a second in **Figures 8A and 8B** is equivalent to 40-milli-second.

Table I.

Compound	Mechanism	Concentration	Beat Rate	Amplitude	Pattern Change
Carbachol	Muscarnic receptor agonist	333 nM	From ~ 80 to ~ 60	From 0.07 to 0.06	Beating rate decreased
Isoproteranol	b2 Adrenergic receptor agonist	4.4 uM	From ~ 65 to 115-136	From 0.19 to 0.16	Beating rate increased

5

To further demonstrate the capabilities of the impedance-based monitoring of cardiomyocyte beating in detecting drugs which may adversely affect heart function, a number of drugs which have been pulled out of the market due to cardiotoxic side effects such as ERG channel inhibition and QT elongation were compiled and tested in a dose-dependent manner. The list of these compounds, their mechanism and adverse side affects are shown in **Table II**. For these tests, mouse ES derived cardiomyocytes were seeded at a final density of 25,000 cells in ACEA E-PLATES and continually monitored by the improved impedance-measurement system.

10 Approximately, 72 hours after cell seeding the fast impedance-measurement system including fast kinetic software and fast measurement hardware circuitry was used to establish a baseline reading of cardiomyocyte beating for each well for about 40 seconds. Subsequently, the cells in each well were treated with the indicated drug and dose shown in **Figure 9**. Similar to the cell index plot shown in **Figures 7C and 7D**, the time resolution between two adjacent points in all the figures in **Figure 9** is 40 milli-seconds. In other words, a second in a figure in **Figure 9** is equivalent to 40-milli-second.

15 **Table II** shows a summary of the results, clearly demonstrating that compounds which have been shown to affect ERG channels do affect various aspects of cardiomyocyte beating and function such as frequency of beating, magnitude of beating. Furthermore, as shown in **Figure 10** some of these compounds can lead to qualitatively different or similar patterns. For example, the compounds E4031, Astemizole and dofetilide which are ERG channel inhibitors do contain patterns with

similar features. In summary these results clearly indicate that using the fast kinetic software along with the RT-CES system do lead to a sensitive and robust readout for cardiomyocyte beating that can also detect drugs which are known to be cardiotoxic.

Similar to the cell index plot shown in **Figures 7C and 7D**, the time resolution

- 5 between two adjacent points in all the figures in **Figure 10** is 40 milli-seconds. In other words, a second in a figure in **Figure 10** is equivalent to 40-milli-second.

Table II.

Compound	Mechanism	Concentration	Beat Rate	Amplitude	Pattern Change
Astemizole	anti-histamine	400 nM	0	<0.01	From 68-72 bpm to irregular being to beating stopped
Terfenadine	anti-histamine	200 nM	0	<0.01	From 71-78 bpm to irregular beating to beating stopped
Erythromycin	anti-biotic	13.3 uM	From ~ 60 to ~ 80	0.09 to 0.07	No pattern change
Moxifloxacin	anti-biotic	20 uM	From ~ 80 to ~73	From 0.055 to 0.060	No pattern change
Pentamidine	anti-infective	20 uM	From 71~78 to ~71	From 0.24 to 0.21	No pattern change
Amitriptyline	Serotonergic Inhibitor	4.4 uM	From ~70 to ~ 90	From 0.22 to 0.17	No pattern change
Verpamil	Ca channel blocker	130 nM	From ~ 65 to 0	From 0.13 to 0.06	From ~ 65 bpm to only occasional single beating.
Rosglitazone	PPAR agonist	13.3 uM	From ~79 to ~ 75	From 0.22 to 0.17	No pattern change
Dofetilite		500 nM	From ~ 80 to ~ 180	From 0.09 to 0.02	Pattern changed, much faster
Rofecoxib	COX-2 Inhibitor	13.3 uM	From ~ 68 to ~ 60	From 0.2 to 0.19	No pattern change
Rofecoxib	COX-2 Inhibitor	40 uM	From ~ 62 to ~ 60	From 0.16 to 0.10	Pattern change, no beating after initial treatment, then recovers
Celecoxib	COX-2 Inhibitor	4.4 uM	From ~ 60 to (~20 - ~ 50)	From ~0.2 to ~0.12	Pattern change
Doxirubicin	Anthracycline	40 uM	~ 70	~ 0.16	No pattern change (initially). Beating pattern changes after 2 hrs.
Cyclosporin A	Calcineurin inhibitor	13.3 uM	From ~70 to ~ 80	From 0.18 to 0.15	No pattern change
Propalanol	β 2-adrenergic receptor antagonist	4.4 uM	From ~70 to over 150	From 0.25 to 0.025	Pattern changed, much faster and irregular
Sotalol	K channel inhibitor	13.3 uM	From ~ 80 to ~ 160	From 0.27 to 0.07	Much faster, pattern changed
E4031		120 nM	From 80 to 160	From 0.09 to 0.03	Pattern changed, much faster
DDT		8 uM	From 80 to 140, then to ~ 80	From 0.25 to 0.02	Initially become faster, later irregular beating
PCB		8 uM	From ~ 65 to 0	From 0.2 to 0	Beating stopped
Endosulfan		8 uM	From ~ 75 to 0	From 0.2 to 0	Beating stopped

To further demonstrate the capabilities of the impedance-based monitoring of cardiomyocyte beating in detecting drugs which may adversely affect heart function, compounds at different concentrations were tested to demonstrate the dose-dependent effects of these compounds on cardiomyocytes. Approximately, 72 hours after cell seeding the impedance-measurement system including software and hardware capable of milli-second measurement time resolution was used to establish a baseline reading of cardiomyocyte beating for each well for about 40 seconds. Subsequently, the cells in each well were treated with drugs at different dose concentrations. **Figure 11** shows an example of dose dependent effects of Astemizole on cardiomyocytes beating at different concentrations. At high concentration of 400 nM, Astemizole had such a strong effect on the beating of cardiomyocytes that the beating almost stopped. The effect of Astemizole on the beating of the cardiomyocytes is clearly dose-dependent. At low concentration of 40 pM, its effect on the beating of the cardiomyocytes is small that the cardiomyocyte beating rate was not affected. Similar to the cell index plot shown in **Figures 7C and 7D**, the time resolution between two adjacent points in all the figures in **Figure 11** is 40 milli-seconds. In other words, a second in a figure in **Figure 11** is equivalent to 40-milli-second.

G. Method to assess the developmental and functional consequence of specific gene knockout and transgene expression in ES-derived cardiomyocytes

The ES cells offer a suitable experimental model system that is amenable to genetic manipulation. Therefore, specific genes can be targeted in knockout experiment as well as genes can be expressed in a developmental or stage specific manner under the control of special promoters. The impedance-based measurement system can be used to evaluate the role of these genes in cardiac viability, morphology, development and or beating function. The steps involved in assessing the developmental and functional effect of gene knockout or transgene expression include

- (1) Obtain ES cells harboring specific knockout of genes or which expresses a particular transgene.
- (2) Provide a single-well or multi-well device that comprise microelectrode arrays in well(s) of the device, which can be used for monitoring cell-substrate impedance.

- (3) Optionally coat wells of the device with either fibronectin or other matrix proteins.
- (4) Seed the embryonic stem cells (ES cells) of mammalian origin or adult stem cells of mammalian origin at specific seeding densities to the wells of the device.
- (5) Allow the cells to attach and spread and monitor the growth and viability of the cells using an impedance measurement system.
- (6) After a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitor cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using milli-second kinetic readout to resolve the individual beat cycles of the cells.
- (7) If a particular gene is required for development of cardiomyocytes from ES cells, it is likely that the knockout of that gene will either affect the viability of the cells or block or delay the differentiation of ES cells to cardiomyocytes. Since the impedance-based measurement system is capable of functional monitoring of cardiomyocyte both in long term assays and short term assays, it can be used as a specific way to monitor the effect of either gene knockout or transgene expression on cardiomyocyte function.
- (8) Alternatively, the ES cells can be transfected with specific siRNA to “knockdown” the product of a particular transcript and then monitor cardiomyocyte viability, differentiation and function in vitro using the impedance-based measurement system.

Similar to Section E, the milli-second kinetic readout requires that the impedance measurement system can provide impedance measurement data at milli-second time resolution. In other words, the time difference between two consecutive impedance measurement for a well shall be in the range of milli-seconds (e.g., less than 500 milli-second, less than 300 milli-second, less than 100 milli-second, less than 10 millisecond, or less than 1 millisecond or faster). The milli-second kinetic readout is required to resolve the individual beat cycles of the cells. Thus, the time resolution for the impedance measurement should allow the system to perform measurement at at least two time points for each beat cycle, or at more than two points for each beat cycle.

One example of the impedance measurement systems is an improved, fast impedance-measurement system from ACEA Biosciences, where the device is ACEA E-PLATE in the form of microtiter plates whose wells comprise microelectrode structures. The steps involved in assessing the developmental and functional effect of gene knockout or transgene expression using the fast impedance-measurement system *in vitro* include:

- (1) Obtain ES cells harboring specific knockout of genes or which expresses a particular transgene.
- (2) Follow steps 1-4 of Section D for using the fast impedance-measurement system for measurement of cardiomyocytes function.
- (3) If a particular gene is required for development of cardiomyocytes from ES cells, it is likely that the knockout of that gene will either block or delay the differentiation of ES cells to cardiomyocytes. Since the system is capable of functional monitoring of cardiomyocyte, it can be used as a specific way to monitor the effect of either gene knockout or transgene expression on cardiomyocyte function.
- (4) Alternatively, the ES cells can be transfected with specific siRNA to “knockdown” the product of a particular transcript and then monitor cardiomyocyte differentiation and function *in vitro* using the fast impedance-measurement system.

H. Devices and method for extracellular recording

Devices and systems of the present invention permit extracellular recording of cell populations using a variety of technical approaches. Extracellular recording may be performed alone or together with impedance monitoring of cells. As discussed in prior sections, while the present device is suitable for performing extracellular recording of various cells and/or tissues, the present invention is particularly useful for the extracellular recording of cardiomyocyte cells, cardiomyocyte precursor cells, as well as tissues that contains such cells.

The devices and systems of the present invention provide structural configurations that functionally establish cell-free zones to prevent direct interaction between cells and reference electrodes, provide enhanced measurement reliability and accuracy, and

bridge the current gap between impedance monitoring and extracellular recording of cells.

For instance, in a first approach, the devices and systems provide a cell-free zone for the positioning of a reference electrode. Accordingly, while traditional systems often require stringent physical parameters for the reference electrode for enhanced measurement, such as stringent tolerances related to surface area, resistance, impedance and the like, present invention provides an alternative solution.

Specifically, in one embodiment, a device for performing extracellular recording of cells, such as cardiomyocytes, is provided which includes a nonconductive substrate forming or provided as a base of one or more wells; an extracellular recording electrode positioned on the substrate within the well, wherein the recording electrode is accessible to cells when a cell sample is added to the device; and a reference electrode positioned within the well in a cell-free zone, the cell-free zone is characterized as free from contact with cells when the cell sample is added to the device, thereby preventing contact between cells and the reference electrode. That is, the reference electrode is a cell-free electrode during operation.

Extracellular recording is conducted by amplifying and recording electrical voltage signals between the recording electrodes and reference electrodes. Such electrical voltages are induced on the electrodes as a result of ionic current or movement through cell culture media or solution supporting the cells during the experiment as a result of opening and/or closing of different ion channels across cell membrane during the action potential duration. Generally, it is desirable and it is recognized for the reference electrodes to have small electrode impedances. The small electrode impedance is achieved by using reference electrodes with large effective surface areas by increasing the ratio of the surface area of the reference electrodes to that of recording electrode by a factor of a hundred, even thousands of times. **Figure 15** shows a schematic representation of such electrode pairs placed on a non-conductive substrate, including a small area recording electrode **1501** and a large area reference electrode **1502**. For clarity, the connection pads on the substrate for connecting the recording electrode and reference electrode to external circuitry are not shown in **Figure 15**. For example, in previous extracellular recording devices, the recording electrodes and reference electrodes are all incorporated onto a substrate surface within

a fluid-container well where the recording electrodes are of simple electrode geometry (e.g. circle, or square) having a diameter of 10 – 100 microns in diameter whereas the reference electrodes would have much larger surface areas.

5 For the present invention, it is recognized that the extracellular recorded voltage signals are recorded as the difference in the electrical potentials between the recording electrode and reference electrode. In order to achieve improved consistency and reproducibility of the recorded voltage signals, it is desirable to minimize the contribution of any electrical signal from the reference electrode to the recorded
10 voltage signals and to ensure that the majority, if not all, of the recording voltage signals are derived from that on the recording electrode. As mentioned above, the previous extracellular recording devices are designed to this goal by reducing the electrode impedance of the reference electrode through using reference electrode of a large surface area and so minimizing the voltage signals on the reference electrode.

15

In one aspect, the present invention is directed to a device for extracellular recording of cells, the device comprising: a nonconductive substrate forming a base of one or more wells; a recording electrode positioned on the substrate within the well, wherein the recording electrode is accessible to cells when a cell sample is added to the
20 device; and a reference electrode positioned within the well in a cell-free zone, the cell-free zone characterized as free from contact with cells when the cell sample is added to the device, thereby preventing contact between cells and the reference electrode. In one embodiment, the recording electrode has a diameter from about 10 um to about 200 um. In another embodiment, the recording electrode comprises an
25 electrode structure comprising a plurality of electrode elements. In preferred embodiments, the reference electrode is positioned on the substrate in the cell-free zone.

Now with reference to particular configurations, the device for extracellular recording
30 of the cells is designed and constructed so that the reference electrode is positioned in the well in a cell-free zone so that when the cells are added to the device, the cells would not be in contact with the reference electrode. In comparison with the situation where the cells are in contact with the reference electrode, no-cell-contact

with the reference electrode would significantly reduce the contribution of the electrical signals from the reference electrode so that the amplified and recorded voltage signals would mainly come from the recording electrode. Consider the following. When the cells are in contact with the reference electrodes, the ionic current and ionic movement in the regions close to the cells due to the opening and/or closing of ion channels (and/or other ion transporters) in the cell membrane would directly cause an electrical potential signal on the close-by reference electrode since these reference electrodes are in direct contact with the cells. On the other hand, when there is no cell in direct contact with the reference electrode, only the ionic current and ionic movement due to the opening and/or closing of the ion channels in the membranes of other cells may result in small, if any, electrical potential on the reference electrode since these cells are not in contact with the reference electrode. The induced electrical potential on the reference electrode due to such no-contact-cells located at other regions of the wells would be significantly smaller than that induced by the cells in direct contact with reference electrode, because the ionic movement/current in the media would be much smaller in the reference electrode region which is away from the cells.

There are different approaches to position the reference electrode within the well in a cell-free zone to prevent contact between cells and the reference electrodes. In one embodiment, the reference electrode is positioned on the substrate forming or provided as the base of the wells to which the cells are added. The reference electrode is located in a region that is separated by a barrier from the region containing the recording electrode. Preferably, for the device of the present invention, the reference electrode may be separated by a barrier from the recording electrode so that when the cells are added to the wells containing the recording electrode, the cells will be prevented from landing on and contacting with the reference electrode. When the cells are added to the recording electrode region, the cells are prevented from moving to the reference electrode region and prevented from contacting the reference electrode. Example of this embodiment is shown in **Figure 33** where both top view and cross-sectional view of a device of the present invention are shown. Here, the recording electrode **3301** and reference electrode **3302** are incorporated on the substrate **3304**. The barrier **3303** is placed inside the well **3305**, separating the recording electrode and reference electrode. In use, the cell culture

media (or appropriate solutions for supporting the cells during the experiment) containing the cells is added first to the left half of the well **3305** to the recording electrode region, ensuring that the solution would not overflow above the barrier to the reference electrode. After all the cells settle down to the substrate (which may
5 take some minutes to typically less than an hour), the culture media (or appropriate solutions for supporting the cells during the experiment) not-containing the cells can be added to the right half of the well and sufficient media (or or appropriate solutions for supporting the cells during the experiment) is added so that the media would overflow above the barrier, the final media height in the well would be above the
10 barrier and there would be electrical connection path between the recording electrode and reference electrode through the media. Note that for simplicity of the figure, the electrical connection traces for the reference electrode and the recording electrode to the connection pads and the connection pads on the substrate are not shown in **Figure 33**. In a related embodiment, the barrier could be provided in a configuration having
15 a series of permeable apertures that selectively permit flow of media while selectively preventing the migration or movement of cells into the cell-free zone; however, such a configuration may lend to clogging of the apertures by blocking cells. In an exemplary embodiment of this approach, the reference electrode may be located under the permeable aperture structure so that the media would flow to the reference
20 electrodes whilst the cells would not.

In another embodiment, the barrier is in a form of a plug that is made of biocompatible materials and can be placed directly above and on the reference electrodes. When the cells are added to the well, the cells are prevented by such a
25 plug to be in contact with the reference electrode and the cells would land on other regions of the substrate, including the recording electrode region. After the cells settle down and adhere to the substrate of the well, the plug is removed and the media or solution in the well would move into the reference electrode region. A complete circuit is formed between the recording electrode and the reference electrode through
30 the media or solution in the well.

Barriers may take alternative forms. For example, barriers may include ledges, walls, troughs and the like to act as a structural or physical barrier to cells. In some instances, a cell repulsive gel is applied to the substrate to define the cell-free zone.

The barrier should however permit the medium to contact the reference electrode such that the recording and reference electrode are operably linked through a conductive media. Barriers may be permanently fixed to the device or may be removable as the configuration permits. Similarly, the barrier may be formed from any suitable material and have any suitable dimensions consistent with isolating the reference electrode from cells, while obtaining extracellular recording measurements. In some embodiments the barrier is constructed from the same material as the nonconductive substrate.

In another embodiment, the reference electrode is positioned on a different plane from the recording electrode, ensuring that the cells added to the recording electrode region would not be in contact with the reference electrode. In such an embodiment the cell-free zone may be a raised surface to prevent cell migration or spreading onto the cell-free zone. In other embodiments an angled surface prevents migration upward along the angled surface and thus provides a suitable cell-free zone towards the top of a slope. In another embodiment, the cell-free zone is a region encapsulated by a porous covering, which permits passage of medium while preventing access by cells.

In still another embodiment, the reference electrode is an external electrode free from contact with the substrate to which the recording electrode is positioned. For instance, a cell-free zone may be spatially positioned remote from the substrate but within a volume of the well. The schematic representation of such a device is shown in **Figure 34**. A recording electrode **3401** is positioned on the substrate **3404**. The reference electrode is an external wire electrode (e.g. gold wire, or Silver-silver chloride wire electrode if they are placed into the well for very short time, not causing cytotoxic effects) **3402**. In **Figure 34**, an impedance electrode array **3406** is also positioned on the substrate **3404**. The electrode array **3406** is of straight-line, interdigitated electrode geometry and comprises two electrode structures, each of which comprise multiple electrode elements. In use, when the cells are added to the well **3405** containing the recording electrode **3401** and impedance measurement electrode array **3406**, the external reference electrode **3402** can be outside of the well. After the cells are added to the wells and are settled down to the bottom, the external reference electrode **3402** can be added to the well manually or automatically and the reference electrode would not be in contact with the substrate of the well and not be in contact with the cells on the substrate. In some embodiments, the external reference

electrode **3402** is fabricated to a lid or cover which may be placed over top of the well.

Thus, a cell-free zone may be spatially positioned away from the substrate. In such a configuration the cell-free zone may occupy a volume of the well that does not contact the substrate, yet does contact medium when added to the device.

Accordingly, while cells do not contact the cell free zone, an electrically conductive media may still ensure operable contact between an extracellular recording electrode and a reference electrode.

For extracellular recording, the recording electrode is generally of simple geometry and consists of a single electrode element such as a circle, a square or some other geometry. The size of such recording electrode typically ranges from about 10-30 to about 100 micron in a diameter. Such small electrode geometry has advantages of recording the electrical potential generated by a small number of the cells located on the recording electrodes. Action potentials from such a small number of the cells tend to be synchronized or nearly synchronized, allowing for a better time resolution for recording extracellular potential and for resolving different features of the recorded potential. However, one limitation of such extra-cellular recording is that due to small area of such electrodes, there tends to be large variations in recorded signals between different recorded electrodes of the same geometry in the same wells (if multiple recording electrodes are positioned inside a single well) or different wells. In particular, if insufficient number of the cells is added to the well to cover all the recording electrodes, it is possible that some recording electrodes may not show any recorded signal or only show recorded signals of very small magnitude.

Furthermore, depending on exact distribution or locations of the cells on the recording electrodes, different recording electrodes may show significantly different extracellular potential waveforms. For this reason, many existing devices for extracellular recording comprise multiple small-area recording electrodes. Extracellular potentials from each such recording electrode are amplified and recorded separately. The user would pick and choose appropriate signal waveforms recorded for some individual recording electrodes for data analysis.

To overcome such a limitation, in some embodiments of the present invention, electrode structures having larger surface areas and including multiple electrode elements are used as recording electrodes. The advantage of recording extracellular potential with such electrode structures is that the extracellular potential is an
5 integration of the potentials from all the cells on the electrode array and would be more reproducible between different wells. The variation in recorded signals between different electrode structures from different wells would be smaller than that recorded with small, single recording electrode elements. For such devices, each well may comprise a single electrode structure, or two electrode structures. In
10 preferred embodiment, each well comprises a single electrode structure consisting of multiple electrode elements.

Thus, the extracellular recording electrode may be provided in a variety of configurations. In some instances the recording electrode includes a unitary, circular
15 or unbranched configuration. In other embodiments the extracellular recording includes an electrode structure, which itself may include a plurality of electrode elements. Exemplary electrode structure and electrode element configurations may be found in US patent nos US 7,470,533; US 4,459,303; US 7,192,752; US 7,560,269; and US 7,468,255, each of which is incorporated by reference. However, the majority
20 of the above listed patents refer to arrays of electrodes having at least two complementary electrode structures. Thus, when considering the configurations for use as an extracellular recording electrode structure or element, the skilled artisan would typically provide one half of the array as set forth in the patents.

Figure 13 shows more specific examples of such electrode structures that could be used as recording electrode for recording extracellular potentials. In **Figure 13A**, a single electrode structure is shown, comprising multiple rectangular electrode
25 elements **1301** that are connected together with an electrode bus **1303**. Similarly, **Figure 13B** comprises a single electrode structure, comprising multiple circle-on-line electrode elements **1311** that are connected together with an electrode bus **1313**. For
30 **Figure 13C**, two electrode structures are shown, one electrode structure comprising multiple electrode elements **1331** and having electrode bus **1333**, and the other electrode structure comprising electrode elements **1332** and having electrode bus **1335**. The extracellular recording can be achieved using either electrode structure or

both electrode structures connected together. **Figures 22 and 23** show example of recording extracellular potentials using circle-on-line electrode structures as recording electrode and external wire electrode as reference electrode. Whilst the recorded waveform is different from those obtained with small recording electrode, the recorded signals are very reproducible between different wells.

By way of yet another example, , a device for performing extracellular recordings of excitable cells in vitro, may include, a) a nonconductive substrate; b) one or more wells on the substrate; c) one or more electrode structures fabricated on the substrate, each electrode structure is associated with one of the one or more wells; and d) one or more reference electrodes external to the substrate or free from contact with the substrate, each of which can be inserted into one of the one or more wells, wherein for each of the one or wells, the corresponding electrode structure and reference electrode form an electrode pair. In further embodiments, the substrate has a surface suitable for attachment of excitable cells, wherein the attachment of excitable cells on the substrate can result in detectable extracellular recording potentials between each of electrode pair. **Figure 12** shows another illustrate example of such electrode pair, comprising a recording electrode structure on the substrate and a reference electrode external to the substrate. In a particularly preferred embodiment, each electrode structure comprises multiple electrode elements. Use of such a configuration is believed to increase the sampling size and thus minimize or reduce variation between cell populations. In another preferred embodiment, each electrode structure comprises multiple electrode elements, forming half of an interdigitated electrode array (**Figure 13A**). In another preferred embodiment, each electrode structure comprises multiple electrode elements so that these electrode elements form a complete interdigitated electrode array (**13C**) wherein during extracellular recording the electrode elements within entire interdigitated electrode array are connected together. In another preferred embodiment, each electrode structure comprises multiple electrode elements which have different electrode element shapes, including rectangular shape, or sinusoidal shape, spiral or circle-on-line shape. **Figure 13** shows examples of electrode structures having multiple electrode elements that can be used for extracellular recording where **13A, 13B, 13C, 13D, 13E and 13F** are the rectangular shape, circle-on-line shape, a complete interdigitated electrode array, sinusoidal shape, castellated shapes and a complete circle-on-line electrode array,

respectively. **Figure 14** shows the examples of such electrode structures for extracellular recording where a recording electrode structure and a reference electrode are incorporated on the substrate to form a pair of electrodes. Preferably, the reference electrode is separated from the recording electrode by a barrier to the cells so that the reference electrode is in a cell-free zone when the cells are added to the electrodes. Clearly, these electrode structures are significantly different from microelectrode arrays formed by simple electrodes such as circle-shaped electrodes and/or square-shaped electrodes currently used in the field of extracellular recording.

10 In another preferred embodiment of the device for performing extracellular recording, each electrode structure occupies a substantial percentage of surface area of the well that the electrode structure is associated with. In some preferred embodiments, percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 5%. Preferably, percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 10%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 20%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 30%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 50%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 70%. More preferably, the percentage of surface area of the well at the bottom being occupied by the electrode structures is more than 85%. Clearly, such large-surface-area electrodes are significantly different from microelectrode arrays currently used in the field of extracellular recording, where recording electrodes typically have a round disc shape or a square shape of smaller size with typical dimension of 10 to 100 micron in diameter..

30 In another aspect of the present invention, a method performing extracellular recording of excitable cells comprises, providing a device of the present invention, adding excitable cells to the wells; providing an extra-cellular-recording amplifier that can measure and record voltage signals at microvolt levels, connecting electrodes on the devices to the extra-cellular-recording amplifier; monitoring and recording extracellular potentials at the electrodes of the devices. Preferably, the method

further comprises analyzing recorded extracellular potential waveforms. More preferably, the method further comprises adding a compound to the well, measuring and recording extracellular potentials prior to and after the compound addition; analyzing extracellular potential waveforms.

5

I. Devices and method for parallel extracellular recording and cell impedance measurement

In still another aspect of the present invention, a device allowing for parallel
10 extracellular recording and cell impedance measurement is provided. Thus, a single device or system is capable of performing both impedance monitoring of cells and extracellular recording. Thus, the device may include a means for cell impedance measuring and a means for extracellular recording operably coupled to the same substrate. In some instances, impedance monitoring occurs before extracellular
15 recording measurements. Such an approach may permit monitoring, determining or confirming desired cell viability, morphology and the like as set forth in prior sections prior to initiating extracellular recording assays. Thus, impedance monitoring permits the user to confirm cellular phenotype or attributes prior to initiating extracellular recording. In other applications, the impedance measurement permits the monitoring
20 of the beating of cardiomyocytes, the monitoring of the change to cell morphology, cell adhesion, cell viability or other properties after a compound treatment. Further, impedance monitoring may be conducted while performing extracellular recording or after extracellular recording. Impedance monitoring may be used to monitor cell beating, viability, morphology and the like. While impedance monitoring and
25 extracellular recording may be performed over the same time period, the actual measurements may be obtained at different time points. That is, a switching means may permit the device to switch from an impedance monitoring mode to an extracellular recording mode. The combined approach is facilitated in part due to the increased impedance based time resolution. That is, impedance monitoring with
30 millisecond time resolution permits such measurements to be conducted throughout a variety of time windows, without adversely affecting extracellular recording measurements.

The skilled artisan will appreciate parallel extracellular recording may be performed using a variety of configurations provided herein. In a first example, parallel recording is performed using a device that includes both a pair of electrodes for impedance monitoring and a pair of electrodes for extracellular recording, though
5 pairs of electrodes may include a third or more electrode. Each pair is selectively operated via a switching means able to activate either an impedance monitoring mode, which allows for the connection of the impedance electrodes to external impedance measurement circuitry; or extracellular recording mode, which allows for the connection of the extracellular recording electrode and reference electrode to external
10 voltage amplifier and signal measurement and recording circuits. In preferred embodiments, the switch is a programmed switch with adjustable parameters through a computer interface. Thus, in one example, a device for parallel impedance monitoring and extracellular recording of cells is provided, wherein the device includes: a nonconductive substrate forming or provided as a base of one or more
15 wells; at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate, wherein the at least two impedance electrodes are accessible to cells when a cell sample is added to the device; an extracellular recording electrode positioned on the substrate within the well, wherein the recording electrode is
20 accessible to cells when the cell sample is added to the device; and a reference electrode positioned within the well. In the preferred mode, the reference electrode is positioned in a cell-free zone, which is characterized as free from contact with cells when the cell sample is added to the device. When in impedance mode, the pair of impedance electrodes permits impedance monitoring of cells. When in extracellular
25 recording mode the extracellular recording electrode and reference electrode form a pair of electrodes for extracellular recording measurements. In a related embodiment, a device is provided that includes a nonconductive substrate, having a surface suitable for attachment of excitable cells, one or more wells on the substrate; for each well, a pair of impedance measurement electrodes, wherein the attachment of excitable cells
30 on the substrate can result in a detectable impedance change; for each well, a pair of extra-cellular recording electrodes comprising a recording electrode and a reference electrode, wherein the attachment of excitable cells on the substrate can result in a detectable extra-cellular recording potentials between the recording-electrode and reference-electrode pair. Preferably, the pair of impedance measurement electrodes

is located on the substrate. Preferably the extracellular recording electrodes are located on the substrate and in the same plane as the impedance electrodes. Also, preferably in such a configuration, the reference electrode is provided in a cell-free zone. The cell-free zone prevents direct contact between cells and the reference electrode. In some instances, a physical barrier prevents contact between cells and the reference electrode. In other instances, the cell-free zone is provided on a surface that is not planar with the impedance monitoring electrodes such that the reference electrode is free from contact with cells added to the device. In still further instances, the cell-free zone is spatially positioned within the well but does not contact the substrate. In such instances, one recording electrode may be located on the substrate and another electrode may be located external to the substrate, but within a volume of the well. Positioning the reference electrode in the volume of the well free from contact with cells and the substrate permits electrically conductive medium to complete an electrical circuit between the recording electrode and reference electrode; however direct contact between cells and the reference electrode may be avoided.

In some embodiments, the recording electrode has a diameter from about 10 μm to about 200 μm . In other embodiments, the recording electrode comprises an electrode structure comprising a plurality of electrode elements

In other configurations, one of the impedance monitoring electrodes is provided as an extracellular recording electrode. **Figure 16** shows an example of such device where in one well, a pair of impedance measurement electrodes is located on the substrate and one of the impedance measurement electrode structures is used as an extracellular recording electrode, together with an externally applied reference electrode. In such a configuration a switching means may selectively switch between coupling signal between the two impedance electrodes in an impedance mode and between the impedance electrode and a reference electrode in an extracellular recording mode. More particularly, in one embodiment the device for parallel extracellular recording includes a nonconductive substrate forming or provided as a base of one or more wells; at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate; and a reference electrode, wherein a first impedance electrode from the at least two impedance electrodes is electrically coupled to the

reference electrode for performing an extracellular recording measurement at the first impedance electrode. In such configurations, preferably the at least two impedance electrodes each have the same surface area and each comprise an electrode structure comprising a plurality of electrode elements. While in some embodiments the
5 reference electrode is positioned on the substrate in other embodiments the reference electrode is an external electrode free from contact with the non-conductive substrate.

In still another aspect of the present invention a device for parallel extracellular recording includes a nonconductive substrate forming or provided as a base of one or
10 more wells; at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate; and an extracellular recording electrode, wherein a first impedance electrode from the at least two impedance electrodes is electrically coupled to the extracellular recording electrode to act as a reference electrode for
15 performing an extracellular recording measurement. That is, the impedance electrode may also operate as a reference electrode for the extracellular recording electrode. In such configurations, preferably, the at least two impedance electrodes each have the same surface area and each comprise an electrode structure comprising a plurality of electrode elements. In one specific embodiment, the recording electrode for
20 extracellular recording is in the form of a unitary electrode structure and the reference electrode is one impedance electrode comprising multiple electrode elements. For the devices of such configuration, the cells would contact the impedance electrodes and contact the reference electrode. This is different from other embodiments of the devices used for extracellular recording described above. The advantage of this
25 approach is that the electrode design for parallel impedance monitoring and extracellular recording is simplified. Instead of using four electrodes with two electrodes for impedance monitoring and two electrodes for extracellular recording, three electrodes are used with one impedance electrode being used for impedance monitoring and used as a reference electrode for extracellular recording. For such
30 configuration, the extracellular recorded voltage signals would have the contributions from extracellular potential from both recording electrode and the reference electrode.

The skilled artisan will recognize the device may be operable linked to a system including an impedance analyzer, extracellular recording amplifier, electronic

switches, electrical noise filtering circuits, switching means capable of switching measurement between the at least two impedance electrodes and the first impedance electrode and the reference electrode and the like. The skilled artisan will also recognize the system may include software to instruct the desired measurements or to set desired parameters for testing or operation.

In still another aspect of the present invention, the method for parallel measurement of cell-substrate impedance and extra-cellular potentials comprises a) providing a device allowing for parallel extracellular recording and cell impedance measurement; b) adding excitable cells to the wells of the device; c) providing an impedance analyzer; d) providing an extracellular potential amplifier; e) connecting electrodes of the devices to the the impedance analyzer; f) connecting electrodes of the devices to the the extra-cellular potential amplifier; g) performing parallel measurement of cell-substrate impedance and extracellular potentials. Preferably, the method further comprises adding compounds to the cells and monitoring cell-substrate impedance and extracellular potentials prior to and after compound addition. Preferably, the method further comprises analyzing measured time dependent impedance responses. Still preferably, the method further comprising analyzing extracellular-recording wave forms.

J. Devices and method for monitoring cardiomyocyte beating using label-free method

In another aspect of the present invention, a label-free method is used to monitor cell-substrate interaction for quantifying and measuring the beating of cardiomyocytes. The label-free method refers to any method that can be monitor or measure the cells without the need of using labeling reagents or molecules. Cell impedance measurement method is one example of label-free approaches. Another label-free method includes the use of Resonant Waveguide Grating (RWG) biosensor (see reference, Y. Yang, Label-Free Cell-Based Assays with Optical Biosensors in Drug Discovery, in *ASSAY and Drug Development Technologies*, Volume 4, Number 5, 2006, 583-595; Y. Yang et al., Label-Free Cell-Based Assays for GPCR Screening, in *Combinatorial Chemistry & High Throughput Screening*, 2008, 11, 357-369; Y.

Yang, Non-invasive Optical Biosensor for Probing Cell Signaling, in Sensors, 2007, Volume 7, 2316-2329).

- An RWG biosensor consists of a substrate (e.g., glass), a waveguide thin film with an embedded grating structure, and a cell layer. The RWG biosensor utilizes the resonant coupling of light into a waveguide by means of a diffraction grating, leading to total internal reflection at the solution-surface interface. This type of biosensor can be used in cell-based assays to monitor changes in cell morphology, cell adhesion or other cell status parameters. The system for using such RWG biosensors can be divided into those based on angle-shift or wavelength-shift measurements. In a wavelength-shift measurement, polarized light covering a range of incident wavelengths with a constant angle is used to illuminate the waveguide; light at specific wavelengths is coupled into and propagates along the waveguide. Alternatively, in angle-shift instruments, the sensor is illuminated with monochromatic light and the angle at which light is resonantly coupled is measured. The resonance conditions are influenced by the physical properties of the cell layer that contacts with the surface of a biosensor (e.g., cell confluency, adhesion and status such as proliferating or quiescent states).
- Such optical biosensor in the form of resonant waveguide, consisting of a substrate with an optical grating and a coating with a high refraction index, may be used as label-free means to monitor the beating of cardiomyocytes by monitoring the change in refractive index upon cell-substrate interaction during cardiomyocyte contraction and relaxation cycle (i.e. beating cycle). The method for such label-free monitoring of beating of cardiomyocytes includes providing a RWG biosensor for monitoring cell status operably connected to angle-shift or wavelength shift measurement system, where the device includes at least two wells optionally coated with fibronectin (or other extracellular matrix proteins) to expedite attachment; adding cells to the at least two wells, where the cells can be mouse or human or other mammalian ES cells destined to differentiate into cardiomyocytes or primary cardiomyocytes isolated directly from the heart of an experimental system including mice, rats, rabbits or dog; optically monitoring the cells of at least two wells at time intervals over a period of time via the measurement of wavelength shift or angle-shift, optionally calculating

average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats.

5 Examples: Extra-cellular Recording and Cell-substrate Impedance Measurement of Stem-cell derived Cardiomyocytes

EXAMPLE 1: Extra-cellular Recording on a disc-shaped microelectrode array

Medium and reagents

10

The standard cell culture medium was Cor.At® Culture Medium (AXIOGENESIS AG, Cologne, Germany) supplemented with 5% of fetal bovine serum (Hyclone, Logan, USA), 100 µg/ml of puromycin. Fibronectin from bovine plasma (1mg/mL solution, Sigma, St. Louis, USA) was used as coating (4 hrs or overnight) material before plating the mouse embryonic stem cell. E4031, a class III antiarrhythmic drug which is a specific antagonist for hERG and EAG like channels was obtained from Sigma (St. Louis, USA).

Cardiomyocyte preparation

20

Cardiomyocytes (Cor.At®, AXIOGENESIS AG, Cologne, Germany) are derived from transgenic mouse embryonic stem cells. Each vial contains 1 million viable Cor.At® cardiomyocytes (> 99.9% pure). These highly purified cardiomyocyte maintain the phenotype of adult mouse cardiomyocyte and express cardiac-specific connexin-43, which is an indication of the ability for excitation-contraction coupling of differentiated cardiomyocyte *in vitro*.

The stem cells were thawed and the suspension drop of cell density is around 10^{6-7} /ml. The thawed stem cells were plated onto fibronectin coated multi-electrodes chip which is embed in PP dish (~130 µl volume) as the bottom. The cell plated chips were placed in 37°C incubator (with 5% CO₂, 95% humidity). The cell culture medium was changed once a day. The plated Cor.At® cell line can be grown up to 21 days post thaw with medium changes every other day.

Multichannel recording

After three to four days growing in 37°C incubator (with 5% CO₂, 95% humidity), the differentiated cells formed a monolayer onto the multi-electrode chip with some congregated cardiomyocyte showing rhythmically self-beating. The earliest onset of self beating was found on the second day after cell plating. The multichannel extracellular recording was carried out on day 3 to day 5 after cell plating using WPI ISO-DAM8A (eight channel module) amplifier (World Precision Instruments, Sarasota, USA) and Dataq DI-0720 data acquisition interface (Dataq Instrument Inc., Akron, USA). The multi-electrode chip contains an array of 30 electrodes (Figure 17), and the diameter of the electrode tip is 50 μm and the interval between each two neighbor electrode tips is 50 μm. The signals were collected via the 4 electrodes in center which were arranged in a rectangle shape (**A2**, **B2**, **C2** and **D2** as shown in **Figure 17**). For extracellular field potential, the field potential is measured between each of the 4-electrodes and an electrical connection that connects to all the other 26 electrodes. The extracellular field potential (FP) data were collected with following parameters being used: Low cut filter: 5 Hz; High cut filter: 1 K Hz; Sampling rate: 5 K Hz; Display sensitivity: 10 K.

Results

General features of field potential

The FP signal is relatively small; the peak to peak amplitude is ≤ 1 mV. During the early process (day 2 to day 4 after cell plating) of growing and differentiating, the self beating frequency of cardiomyocyte cluster was increased from 60 to 90 /min on day 2 (n=4) to 160 to 220 / min on day 4 (n=4), and the beating frequency remain nearly-constant after day4. In the meantime, the duration of FP was reduced and the depolarizing velocity which reflecting the upstroke of the FP was shortened while the amplitude of the FP was increased (**Figure 18**). As shown in **Figure 18**, the electrophysiological features were changed as the mouse derived stem cell grows and develops on day 2 and day 3. The FP amplitude and frequency were increased while the duration and depolarizing velocity were shortened.

Since the extracellular FP is the negative reflection of the intracellular voltage fluctuation, the standard shape of FP waveform consists downward depolarization peak followed by upward repolarization peak. In actual recording, we observed an initial upward peak and downward peak followed by an upward peak (**Figure 18**).

Effect of E4031 on FP

E4031, antiarrhythmic agent, is a specific antagonist for hERG and EAG channel family which participate repolarization during action potential phase 3. So In present study, we picked FP duration as our main target index, and it directly correlated to the “QT interval” in clinical ECG. We found E4031 concentration-dependently delayed the depolarizing velocity and prolonged FP duration in mouse cardiomyocyte (**Figure 19**) with IC_{50} of 405 nM (**Figure 20**). The on-site action of E 4031 on field potential is a slightly slow and it possibly due relatively the low binding affinity of E4031 to its receptor. At a concentration of 75 to 150 nM, E4031 exerted “anti-arrhythmic” action on field potential frequency in the mouse-stem-cell derived cardiomyocytes (**Figure 21**).

20

EXAMPLE 2: Extra-cellular Recording Using one electrode structure on the substrate and one external reference electrode

Figure 22 shows an extra-cellular field potential recording of mouse stem-cell derived cardiomyocytes obtained using a device of the present invention. The circle-on-line electrode array (with circle diameter 90 micron, line width 30 micron, the electrode gap distance 20 micron, see **Figure 13F** for representation of circle-on-line electrodes) consisting of two electrode structures are fabricated on a glass substrate. During extra-cellular field potential recording, one circle-on-line electrode is used as a recording electrode and an external wire electrode is inserted into the well serving as a reference electrode. Cell preparation, reagents, cell culture and extracellular recording setup are the same as those described in to example 1.

Figure 22A shows a field potential (after an amplification of 10,000) before treatment for a 3-day cardiomyocyte culture in a well containing circle-on-line electrodes. Cardiomyocytes were beating at a rate of ~ 91 beats per minute. At the middle point of the **Figure 22A**, E4031 was added to the well. No immediate effect was observed after the treatment with E4031. However, with time, the field potential gradually became irregular. The irregular field potentials at ~ 18 seconds and ~ 3 minutes after treatment were shown on **Figure 22B** and **22C**. At ~ 5.5 minutes after treatment, the field potential magnitude was significantly reduced and later on, no field potential peaks could be detected.

EXAMPLE 3. Parallel cell-impedance measurement and extra-cellular recording for cardiomyocytes prior to and after Quinidine treatment

Figure 23A through **23E** show another example of field potential change for cardiomyocytes at different time points before and after the treatment with 3 uM Quindine, as recorded from a 3-day cardiomyocyte culture in a well containing circle-on-electrodes. Similar to the configuration for data in **Figure 22**, an external wire electrode was inserted to the well serving as a reference electrode. Before treatment, cardiomyocytes were beating at a rate of ~ 72 beats per minute. As shown in **Figure 23 B** through **D**, corresponding to the field potential recorded at 10 seconds, 50 seconds, 3 minutes and 9 minutes after treatment with 3 uM Quindine, Quindine has a dramatic effect on the field potential of the cardiomyocytes.

In parallel, impedance of the cardiomyocytes in such a well is monitored. **Figure 24A** shows the impedance spikes as monitored on the circle-on-line electrode array before treatment. As shown in **Figure 24B** through **D**, corresponding to the impedance spike pattern measured at ~ 1.5 minute, ~ 3 minute and ~ 11 minute after treatment with 3 uM Quindine, impedance-based monitoring can readily detect the change in cardiomyocyte beating function as a result of Quindine treatment. Notably, at ~ 11 minutes after quindine treatment, the amplitude of impedance spikes significantly reduced and the impedance spiking also becomes very irregular.

EXAMPLE 4. Extra-cellular recording for primary cardiomyocytes between a circle-on-line electrode structure and an externally applied reference electrode

Primary cardiomyocytes were prepared from by perfusing sacrificed mice' heart. The primary cardiomyocytes were added to a well containing a circle-on-line electrode array (with circle diameter 90 micron, line width 30 micron, the electrode gap distance 20 micron) on the well bottom. The electrode array is fabricated on a glass substrate. Before seeding the cells, the well was pre-coated with fibronectin. Each well was seeded with about 40,000 cells and was measured between 48 and 72 hrs after seeding. During extra-cellular field potential recording, one circle-on-line electrode structure is used as a recording electrode and an external wire electrode is inserted into the well serving as a reference electrode. The setting for extra-cellular recording is the same as those used in Example 1. **Figure 25** shows a typical field potential from such a primary cardiomyocyte culture.

EXAMPLE 5: Parallel cell-impedance measurement and extra-cellular recording for cardiomyocytes

Figure 27 shows the design of an electrode array used for combined cell-impedance measurement and extra-cellular recording. For this design, the electrode array consists of two extracellular-recording electrodes **2701** and **2702** with circle geometry having diameters of 60 microns, located on either left or right side of, and in the middle section the array. Note that the recording electrode diameter could be varied from being as small as 5 micron to as large as 0.5 mm, even 1 mm, or even multiple millimeters. Electrode array further consists of two electrode structures **2703** and **2704**, each comprising multiple electrode elements. Electrode elements within each electrode structure are of circle-on-line geometry and comprise interconnected circles having a diameter of 90 microns positioned along a straight line of 30 micron width (the electrode gap distance 20 micron, see **Figure 13F** for representation of circle-on-line electrodes). Electrode element **2705** is the first element counting from top for electrode structure **2703**. Electrode element **2706** is the first element counting from top for electrode structure **2704**. Electrode elements on electrode structure **2703** are connected together through electrode bus **2707**, having a width of 0.2 mm, to a connection pad **2709** which is located on the left side of **Figure 27**. Similarly, electrode elements of the electrode structure **2704** are connected together through electrode bus **2708**, having a width of 0.2 mm, to a connection pad **2710** which is

located on the right side of **Figure 27**. The overall electrode area excluding the regions consisting of electrode buses **2707** and **2708** has a diameter of 3.1 mm.

The electrode array of **Figure 27** is fabricated on glass substrate using photolithography methods for pattern-generation of thin gold electrode arrays (gold film thickness of ~ 100 nm on top of a Cr adhesion layer of ~ 10 nm). A bottomless well plate having a well-diameter of 5 mm is assembled to the electrodes-containing glass slides, with the electrode array shown on **Figure 27** being positioned at the central region of the well.

For cell impedance measurement, the impedance between two electrode structures **2703** and **2704** is measured by connecting an ACEA real-time-cell-analysis impedance analyzer capable of millisecond time resolution (e.g., 15 milliseconds or less impedance data update rate for measuring 96 wells simultaneously) to the two connection pads **2709** and **2710**, connected to responding electrode buses **2707** and **2708**, respectively. For extracellular recording (ECR), various recording modes can be used.

(1) ECR mode 1 is used to for monitoring electrode electrical voltage signals between an ECR electrode **2701** (or **2702**) and one (impedance-measurement) electrode structure **2703** (or **2704**) by connecting an ECR connection pad (**2711** or **2712**) and an impedance-measurement electrode connection pad (**2709** or **2710**) to a voltage amplifier. For this mode, extracellular signals from ECR electrodes are measured with the impedance-measurement electrode structure **2703** or **2704** used as “reference electrode”. The impedance-measurement electrode structure **2703** (or **2704**) has a much smaller electrode-impedance due to its much larger surface area (over 100 times larger) than ECR electrodes **2701** (or **2702**).

(2) ECR mode 2 is used for monitoring electrode electrical voltage signals between an ECR electrode **2701** (or **2702**) and an externally applied gold reference electrode (not shown on **Figure 27**) inserted into the well by connecting an ECR connection pad (**2711** or **2712**) and the gold external electrode to a voltage

amplifier. For this mode, extracellular signals from ECR electrodes are measured with the external gold electrode used as “reference electrode”. Such external gold electrode will not have any cardiomyocytes attached and would serve as cell-free reference electrodes.

- (3) ECR mode 3 is used for monitoring electrical voltage signals between impedance-measurement electrode structure **2703** (or **2704**) and an externally applied gold reference electrode (not shown on **Figure 27**) inserted into the well by connecting an impedance-electrode connection pad (**2709** or **2710**) and the external gold electrode to a voltage amplifier. For this mode, extracellular signals from impedance-measurement electrodes are measured with the external gold electrode used as “reference electrode”. Such external gold electrode will not have any cardiomyocytes attached and would serve as cell-free reference electrodes.

Figures 28 and 29 are examples of impedance measurement and extracellular recording of Cor. At cardiomyocytes. Cor. At cells were prepared and cultured using the same method as those described in Example 1. The data on **Figures 28 and 29** is for the Cor. At cells on day 5 after cell seeding into the electrodes-containing wells. **Figure 28** shows the impedance beating result obtained for the Cor. At cells for ~ 12 seconds with time-resolution about 28.8 ms per data point. The impedance data is represented with cell index, a dimension less parameter. **Figure 29** shows the ECR voltage signals for the three different recording modes described above, including ECR electrode vs impedance electrode-structures, ECR electrode vs external gold electrode and impedance electrode-structure vs external gold electrode. The voltage amplifier and other ECR details are the same as those discussed in Example 1. In brief, the multichannel extracellular recording was carried out using WPI ISO-DAM8A (eight channel module) amplifier (World Precision Instruments, Sarasota, USA) and Dataq DI-0720 data acquisition interface (Dataq Instrument Inc., Akron, USA). The extracellular field potential data were collected with following parameters being used: voltage signal gain: 10,000; Low cut filter: 1 Hz; High cut filter: 1 K Hz; Sampling rate: 2-5 K Hz.

EXAMPLE 6: Parallel cell-impedance measurement and extra-cellular recording for cardiomyocytes

5 **Figure 30** shows the design of another electrode array used for combined cell-impedance measurement and extra-cellular recording. For this design, the electrode array consists of two extracellular-recording electrodes 3001 and 3002 with circle geometry having diameters of 60 microns, located in the middle section the array. Note that the recording electrode diameter could be varied from being as small as 5
10 micron to as large as 0.5 mm, even 1 mm, or even multiple millimeters. ECR electrodes **3001** and **3002** are connected to the connection pads **3011** and **3012**, respectively. The electrode array further consists of two electrode structures **3003** and **3004**, each comprising multiple electrode elements. Electrode elements within each electrode structure are of circle-on-arc (curved line) geometry and comprise
15 interconnected circles having a diameter of 90 microns positioned along curve lines of 30 micron width (the electrode gap distance 20 micron, see **Figure 13F** for representation of circle-on-line electrodes). The first electrode element of the electrode structure **3003**, counting from top of the electrode array, has many circles aligned-on-curved-line, forming a semi-circle arc. The first electrode element of the
20 electrode structure **3004**, counting from bottom of the electrode array, has many circles aligned-on-curved line, forming a semi-circle arc. Electrode elements on electrode structure **3005** are connected together through horizontal lines located in the middle of the electrode array to the connection pad **3009** which is located on the left side of the electrode array. Electrode elements on electrode structure **3006** are
25 connected together through horizontal lines located in the middle of the electrode array to the connection pad **3010** which is located on the right side of the electrode array.

The electrode array of **Figure 28** is fabricated on glass substrate using
30 photolithography methods for pattern-generation of thin gold electrode arrays (gold film thickness of ~ 100 nm on top of a Cr adhesion layer of ~ 10 nm). A bottomless well plate having a well-diameter of 5 mm is assembled to the electrodes-containing glass slides, with the electrode array shown on **Figure 30** being positioned at the central region of the well.

For cell impedance measurement, the impedance between two electrode structures **3003** and **3004** is measured by connecting an ACEA real-time-cell-analysis impedance analyzer capable of millisecond time resolution (e.g., 15 milliseconds or less impedance data update rate for measuring 96 wells simultaneously) to the two connection pads **3009** and **3010**, connected to responding electrode structures **3003** and **3004**, respectively. For extracellular recording (ECR), various recording modes can be used.

(1) ECR mode 1 is used for monitoring electrode electrical voltage signals between an ECR electrode **3001** (or **3002**) and one (impedance-measurement) electrode structure **3003** (or **3004**) by connecting an ECR connection pad (**3011** or **3012**) and an impedance-measurement electrode connection pad (**3009** or **3010**) to a voltage amplifier. For this mode, extracellular signals from ECR electrodes are measured with the impedance-measurement electrode structure **3003** or **3004** used as “reference electrode”. The impedance-measurement electrode structure **3003** (or **3004**) has a much smaller electrode-impedance due to its much larger surface area (over 100 times larger) than ECR electrodes **3001** (or **3002**).

(2) ECR mode 2 is used for monitoring electrode electrical voltage signals between an ECR electrode **3001** (or **3002**) and an externally applied gold reference electrode (not shown on **Figure 30**) inserted into the well by connecting an ECR connection pad (**3011** or **3012**) and the gold external electrode to a voltage amplifier. For this mode, extracellular signals from ECR electrodes are measured with the external gold electrode used as “reference electrode”. Such external gold electrode will not have any cardiomyocytes attached and would serve as cell-free reference electrodes.

(3) ECR mode 3 is used for monitoring electrical voltage signals between impedance-measurement electrode structure **3003** (or **3004**) and an externally applied gold reference electrode (not shown on **Figure 30**) inserted into the well by connecting an impedance-electrode connection pad (**3009** or **3010**) and the external gold electrode to a voltage amplifier. For this mode, extracellular signals from impedance-measurement electrodes are measured with the external gold electrode used as “reference electrode”. Such external

gold electrode will not have any cardiomyocytes attached and would serve as cell-free reference electrodes.

Figures 31 and **32** are examples of impedance measurement and extracellular recording of Cor. At cardiomyocytes. Cor. At cells were prepared and cultured using the same method as those described in Example 1. The data on **Figures 31** and **32** is for the Cor. At cells on day 5 after cell seeding into the electrodes-containing wells. **Figure 31** shows the impedance beating result obtained for the Cor. At cells for ~ 12 seconds with time-solution about 28.8 ms per data point. The impedance data is represented with cell index, a dimension less parameter. **Figure 32** shows the ECR voltage signals for the three different recording modes described above, including ECR electrode vs impedance electrode-structures, ECR electrode vs external gold electrode and impedance electrode-structure vs external gold electrode. The voltage amplifier and other ECR details are the same as those discussed in Example 1. In brief, the multichannel extracellular recording was carried out using WPI ISO-DAM8A (eight channel module) amplifier (World Precision Instruments, Sarasota, USA) and Dataq DI-0720 data acquisition interface (Dataq Instrument Inc., Akron, USA). The extracellular field potential data were collected with following parameters being used: voltage signal gain: 10,000; Low cut filter: 1 Hz; High cut filter: 1 K Hz; Sampling rate: 2-5 K Hz.

CLAIMS

What is claimed is:

- 5 1. A device for extracellular recording of cells, the device comprising:
- a) a nonconductive substrate forming a base of one or more wells;
- b) a recording electrode positioned on the substrate within the well,
10 wherein the recording electrode is accessible to cells when a cell sample is added to the device; and
- c) a reference electrode positioned within the well in a cell-free zone, the
15 cell-free zone characterized as free from contact with cells when the cell sample is added to the device, thereby preventing contact between cells and the reference electrode.
- 20 2. The device according to claim 1, wherein the recording electrode has a diameter from about 10 μm to about 200 μm .
3. The device according to claim 1, wherein the recording electrode comprises an electrode structure comprising a plurality of electrode elements.
- 25 4. The device according to claim 1, wherein the reference electrode is positioned on the substrate in the cell-free zone.
5. The device according to claim 4, wherein the cell-free zone is defined by a barrier, which prevents cell access to the cell-free zone.
- 30 6. The device according to claim 1, wherein the cell-free zone is characterized as being on a different plane than the recording electrode, thereby preventing cell migration to the cell free zone.

7. The device according to claim 6, wherein the cell-free zone comprises a surface vertically above the recording electrode.

8. The device according to claim 1, wherein the reference electrode is an external electrode free from contact with the non-conductive substrate.

9. The device according to claim 1, loaded with a cell sample, wherein cells within the cell sample contact the recording electrode but do not contact the reference electrode.

10. The device according to claim 9, wherein the cell sample comprises cardiomyocytes or cardiomyocyte precursor cells.

11. The device according to claim 1, further comprising at least two impedance monitoring electrodes positioned on the nonconductive substrate and the at least two impedance electrodes are accessible to cells.

12. A device for parallel impedance monitoring and extracellular recording of cells, the device comprising:

- a) a nonconductive substrate forming a base of one or more wells;
- b) at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate, wherein the at least two impedance electrodes are accessible to cells when a cell sample is added to the device;
- c) a recording electrode positioned on the substrate within the well, wherein the recording electrode is accessible to cells when the cell sample is added to the device; and
- d) a reference electrode positioned within the well in a cell-free zone, which is characterized as free from contact with cells when the cell sample is added to the device.

13. The device according to claim 12, wherein the at least two impedance electrodes each have the same surface area and each comprises an electrode structure comprising a plurality of electrode elements.

5

14. The device according to claim 12, wherein the reference electrode is deposited on the substrate in the cell-free zone.

15. The device according to claim 12, wherein the cell-free zone is defined
10 by a barrier, which prevents cell access to the cell-free zone.

16. The device according to claim 12, wherein the cell-free zone is characterized as being on a different plane than the recording electrode, thereby preventing cell migration to the cell-free zone.

15

17. The device according to claim 16, wherein the cell free zone comprises a surface vertically above the recording electrode.

18. The device according to claim 12, wherein the reference electrode is an
20 external electrode free from contact with the non-conductive substrate.

19. The device according to claim 12, loaded with a cell sample, wherein cells within the cell sample contact the recording electrode and the at least two impedance electrodes but do not contact the reference electrode.

25

20. The device according to claim 12, operably linked to a system comprising an impedance analyzer, extracellular recording amplifier and switching means capable of switching measurement between the at least two impedance electrodes and the extracellular recording electrode and the reference electrode.

30

21. A device for parallel impedance monitoring and extracellular recording of cells, the device comprising:

a) a nonconductive substrate forming a base of one or more wells;

b) at least two impedance electrodes capable of monitoring impedance of the cells, the at least two impedance electrodes positioned within a well and on the nonconductive substrate;

c) a recording electrode positioned on the substrate within the well; and

d) a reference electrode, wherein the recording electrode is electrically coupled to the reference electrode for performing an extracellular recording measurement at the recording electrode.

22. The device according to claim 21, wherein the at least two impedance electrodes each have the same surface area and each comprises an electrode structure comprising a plurality of electrode elements.

23. The device according to claim 22, wherein the recording electrode is one of the at least two impedance electrodes and the reference electrode is an external electrode free from contact with the non-conductive substrate.

24. The device according to claim 21, wherein said recording electrode has a diameter from about 10 micron to about 200 micron.

25. The device according to claim 21, wherein the reference electrode is one of the at least two impedance electrodes.

26. The device according to claim 21, wherein the reference electrode is positioned on the substrate.

27. The device according to claim 21, wherein the reference electrode is an external electrode free from contact with the non-conductive substrate.

28. The device according to claim 21, loaded with a cell sample, wherein
5 cells within the cell sample contact the at least two impedance electrodes but do not contact the reference electrode.

29. The device according to claim 21, operably linked to a system
comprising an impedance analyzer, extracellular recording amplifier and switching
10 means capable of switching measurement between the at least two impedance electrodes and the recording electrode and the reference electrode.

30. A method of extracellular recording of a cell population, the method
15 comprising:

a) providing the device according to claim 1, operably linked to a voltage amplifier;

20 b) adding a cell sample to the device, wherein the reference electrode remains free from contact with cells from the cell sample;
and

25 c) performing extracellular recording measurements by
amplifying the voltage signals between the recording electrode and the reference electrode and monitoring the amplified signals.

31. The method according to claim 30, wherein the cells comprise cardiomyocytes or cardiomyocyte precursor cells, the method further comprising
30 adding a compound suspected of affecting a beating cycle of the cells.

32. A method of extracellular recording of a cell population, the method comprising:

5 a) providing the device according to claim 12, operably linked to a voltage amplifier and impedance measurement circuitry;

b) adding a cell sample to the device, wherein the reference electrode remains free from contact with cells from the cell sample;

10 c) performing extracellular recording measurements by amplifying the voltage signals between the recording electrode and the reference electrode and monitoring the amplified signals; and

15 d) performing impedance measurements by monitoring impedance between the two impedance electrodes.

33. The method according to claim 32, wherein the cells comprise cardiomyocytes or cardiomyocyte precursor cells, the method further comprising adding a compound suspected of affecting a beating cycle of the cells.

20 34. The method according to claim 32, wherein the impedance is monitored at milli-second time resolution.

25 35. The method according to claim 32, wherein the impedance is monitored at seconds or minutes time resolution.

30 36. The method according to claim 32, further comprising calculating cell index from measured impedance values.

37. A method of extracellular recording and impedance monitoring of a cell population, the method comprising:

- 5 a) providing the device according to claim 21, operably
 connecting the at least two impedance electrodes to an impedance
 measurement circuitry and connecting the recording electrode and the
 reference electrode to a voltage amplifier;
- 10 b) adding a cell sample to the device;
- c) performing extracellular recording measurements by
 amplifying the voltage signals between the recording electrode and the
 reference electrode and monitoring the amplified signals; and
- 15 d) performing impedance measurements by monitoring impedance
 between the two impedance electrodes.

38. The method according to claim 37, wherein the cells comprise
cardiomyocytes or cardiomyocyte precursor cells, the method further comprising
20 adding a compound suspected of affecting a beating cycle of the cells.

39. The method according to claim 37, wherein further the impedance is
monitored at milli-second time resolution.

25 40. The method according to claim 37, wherein further the impedance is
monitored at seconds or minutes time resolution.

41. The method according to claim 37, wherein further comprising calculating
cell index from measured impedance values.

30

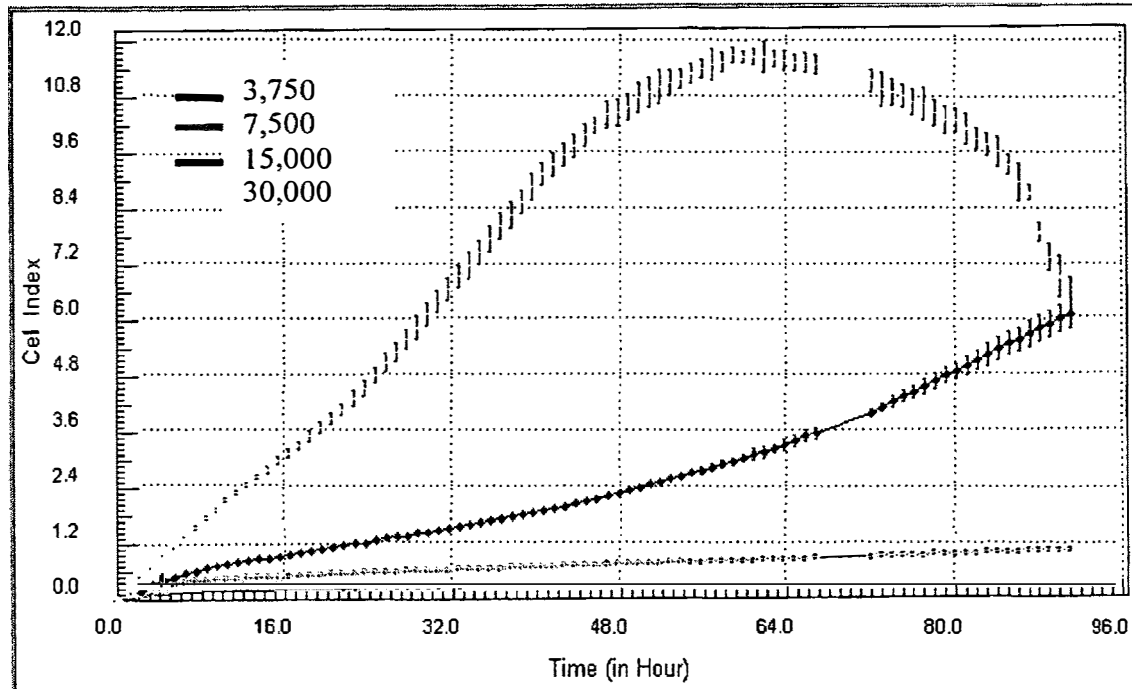
Figure 1.**Titration of ES-derived cardiomyocytes using the ACEA RT-CES system**

Figure 2. Treatment of ES-derived cardiomyocytes with a cytotoxic compound of Sodium dichromate dehydrate.

Fig. 2A

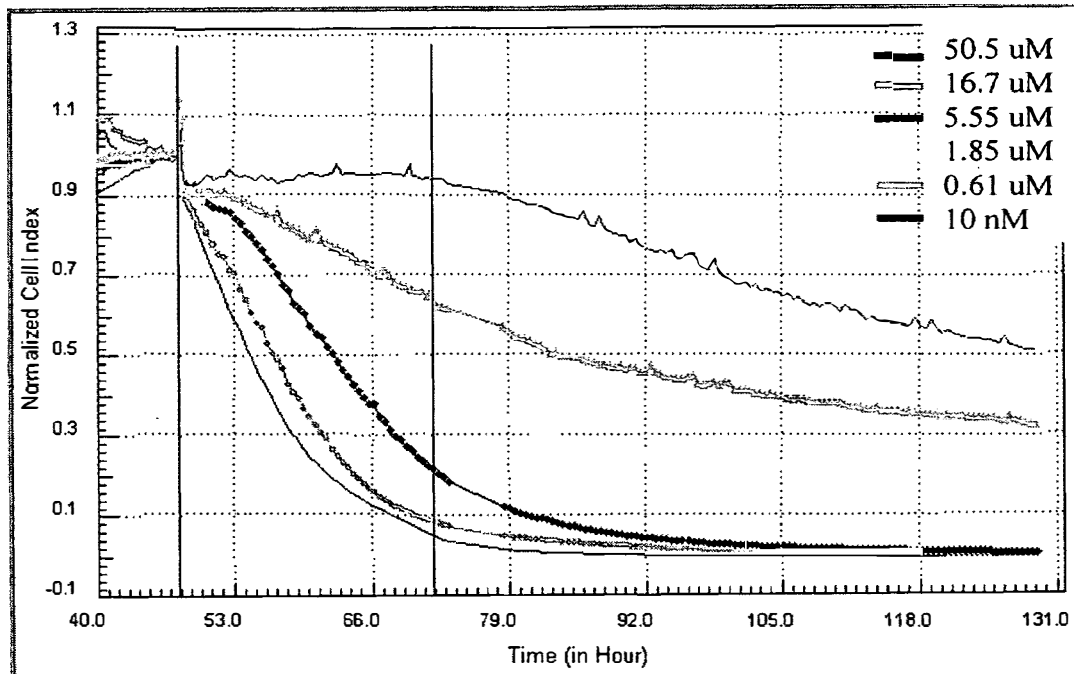


Fig. 2B

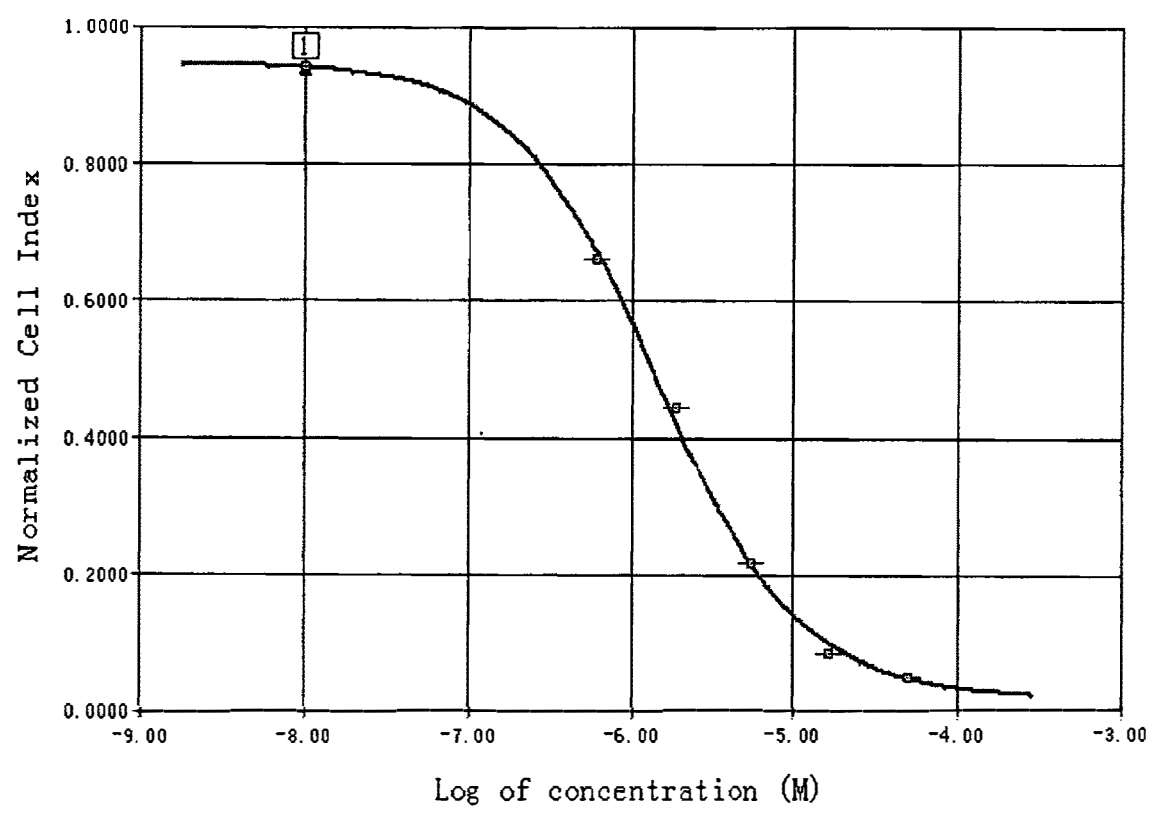


Figure 3. Detection of morphological changes associated with ES-derived cardiomyocytes induced by isoproterenol, a β_2 adrenergic receptor agonist, using the RT-CES system

Fig. 3A.

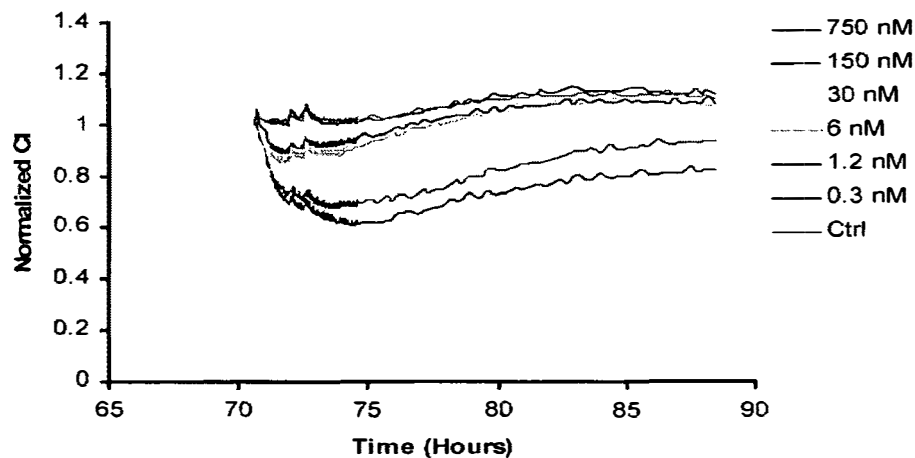


Fig. 3B.

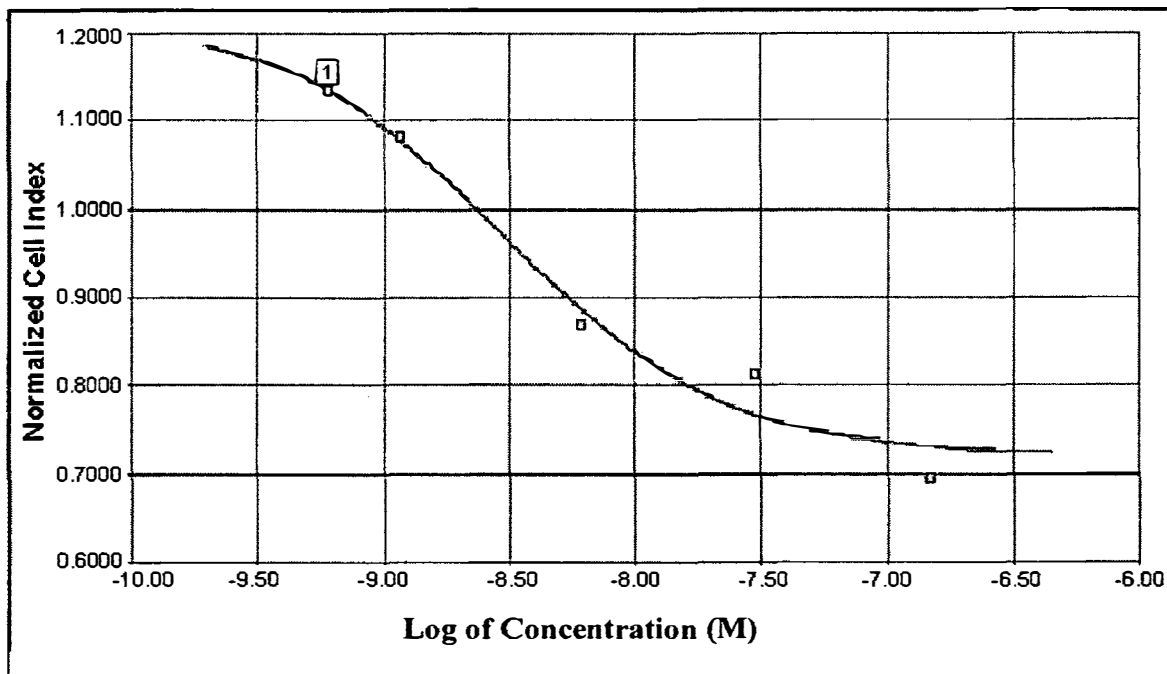


Figure 4.

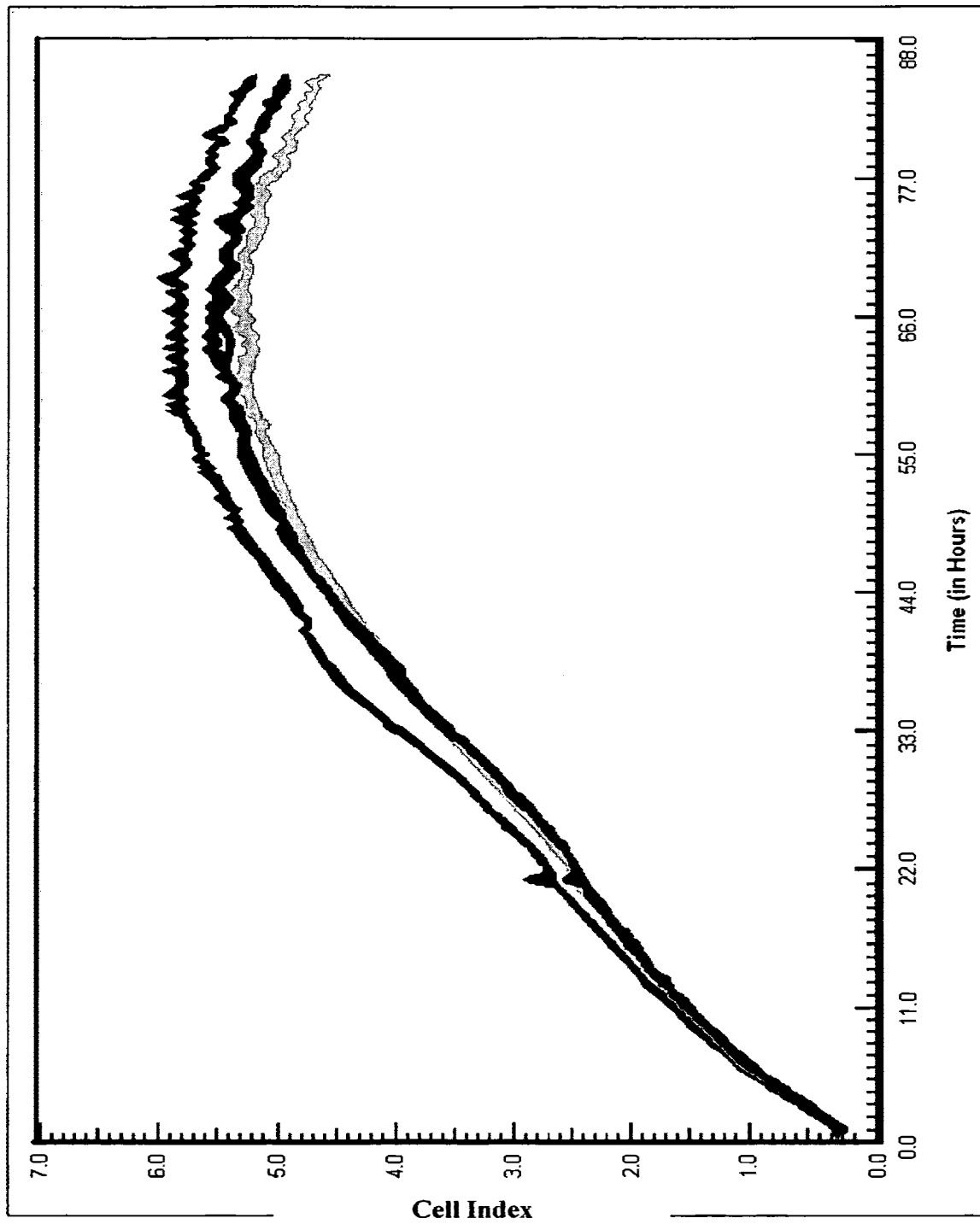


Figure 5. Detection of cardiomyocyte beating using the ACEA RT-CES system at different time periods along the growth and differentiative curve of ES-derived cardiomyocytes seeded in ACEA E-Plates. The time resolution between adjacent points in Figures 5A, 5B and 5C is about 40 milli-seconds. In another words, a second shown in Figures 5A, 5B and 5C is equivalent to 40 milli-seconds.

Fig. 5A

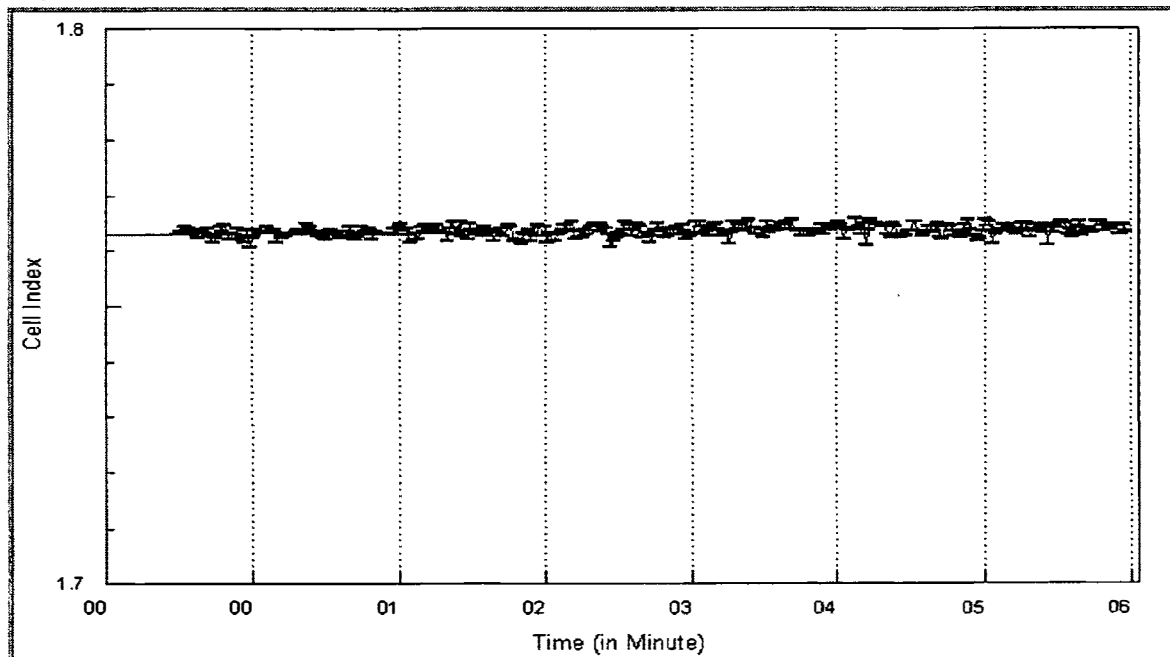


Fig. 5B

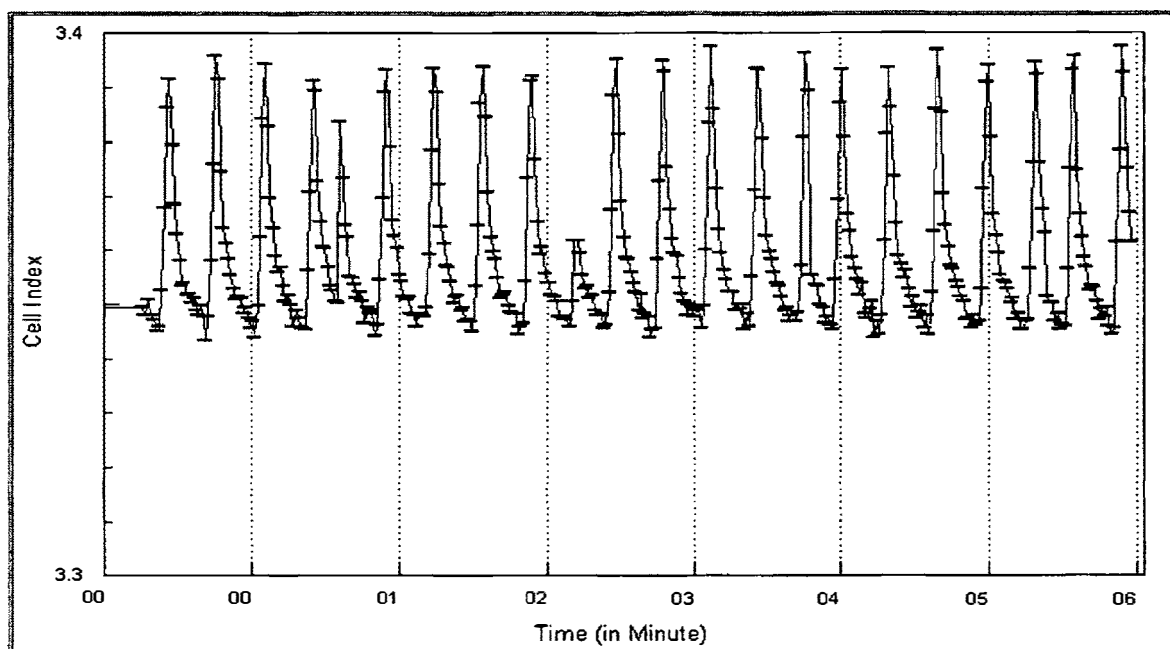


Fig. 5C

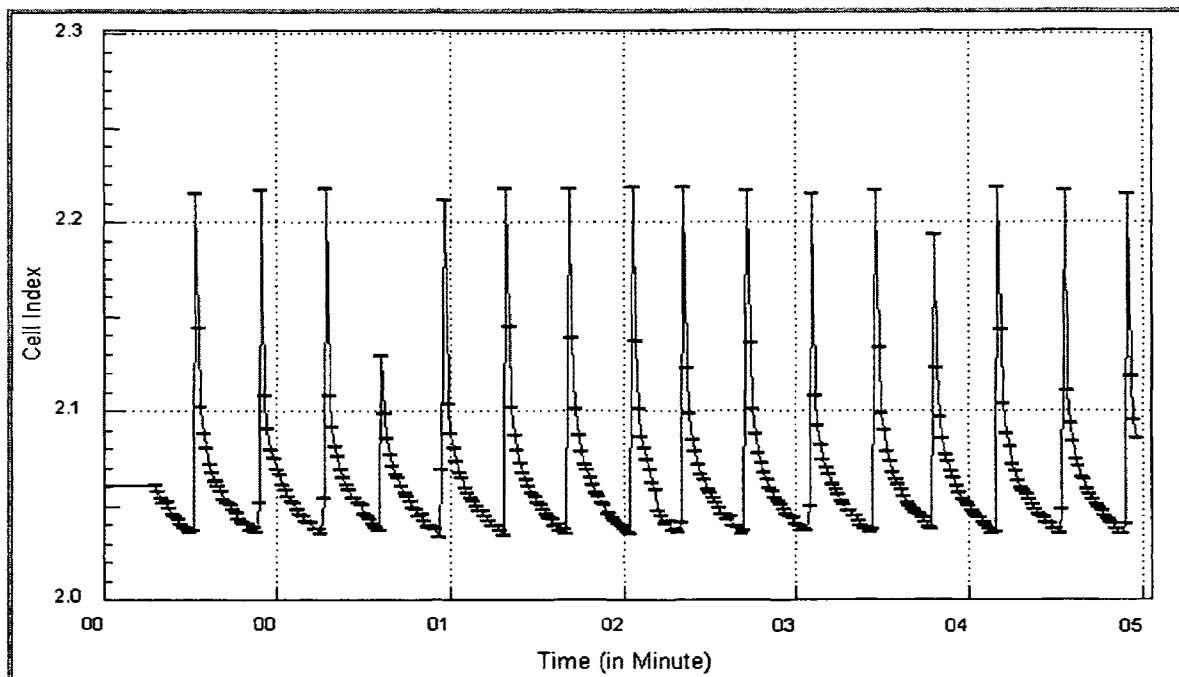


Fig. 5D

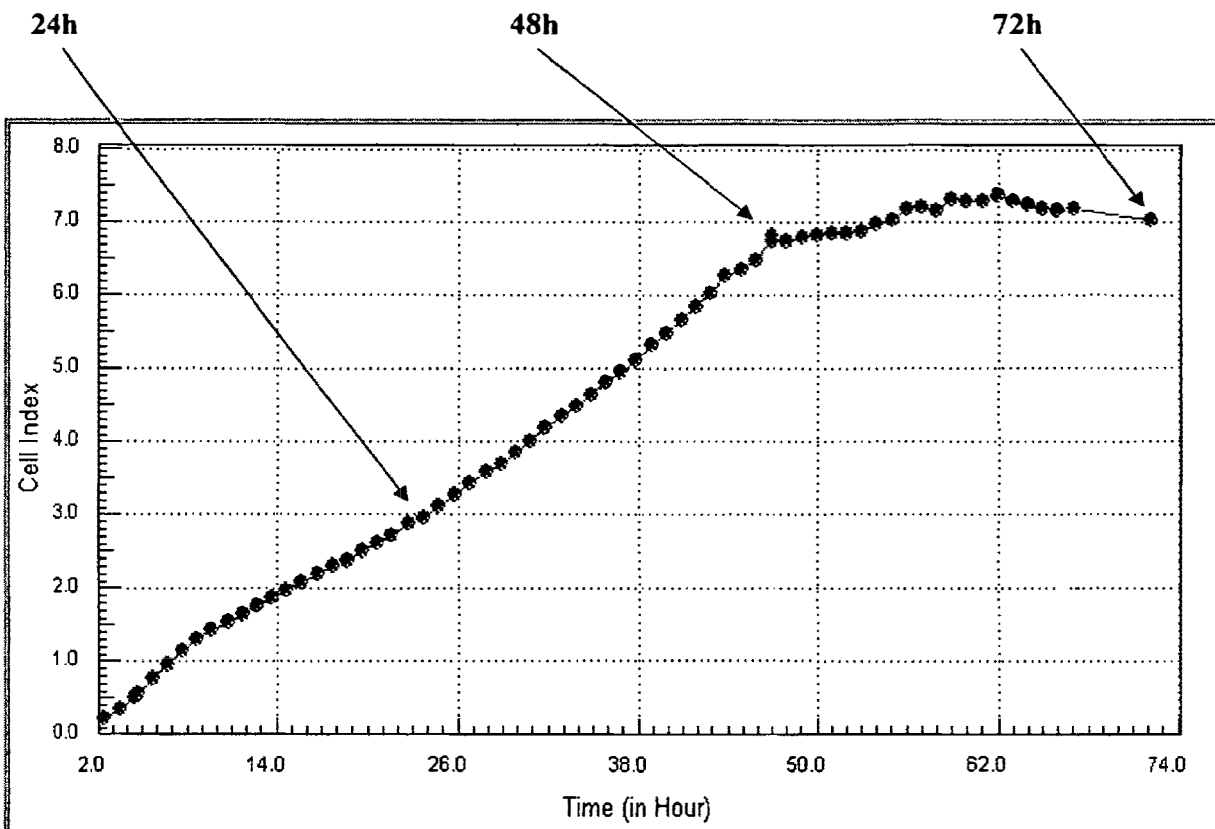


Figure 6A

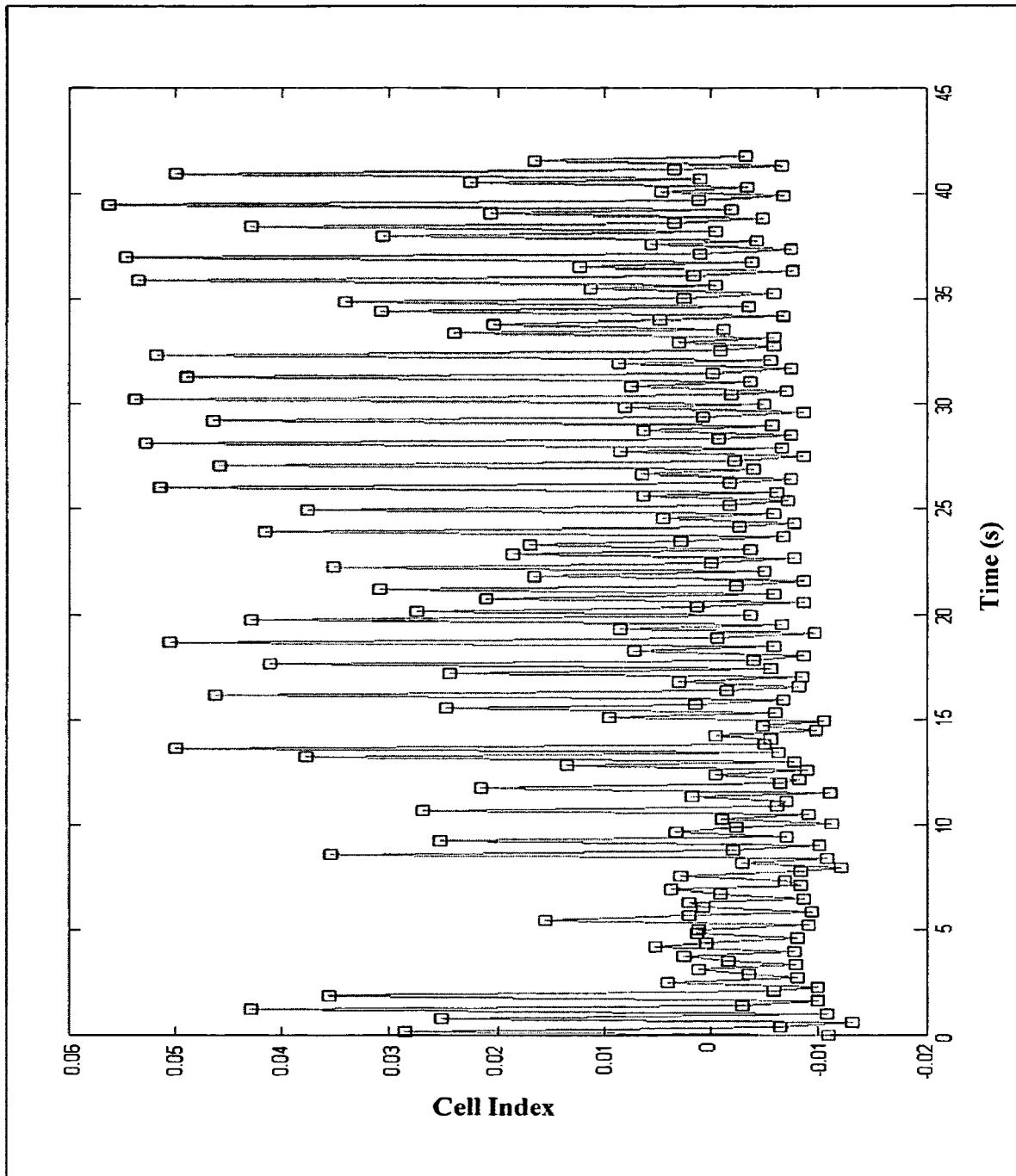


Figure 6B

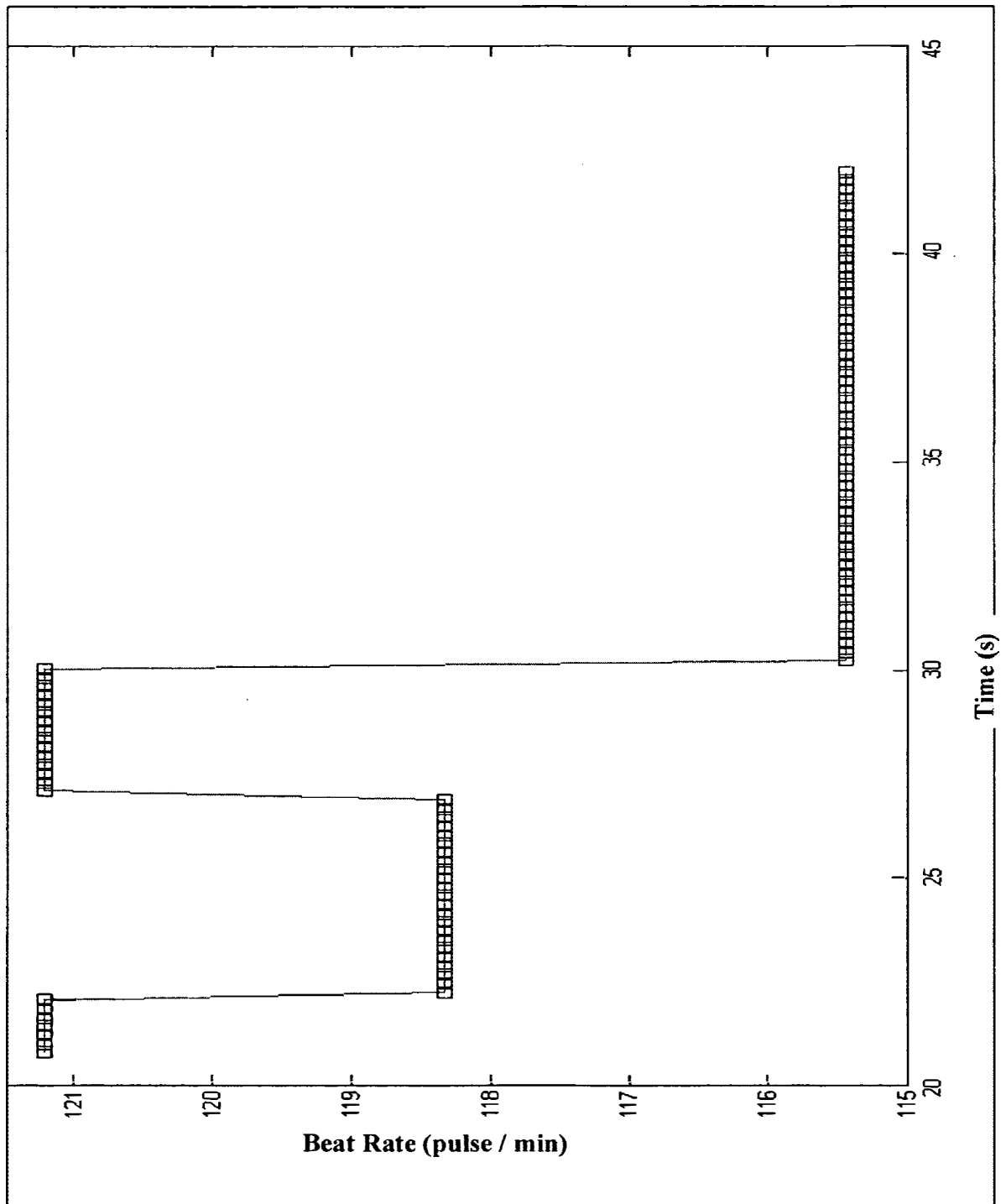


Figure 7. Detection of cardiomyocyte beating using the ACEA RT-CES system for the mouse cardiomyocytes treated with 4.4 μ M sotalol. In Figures 7C and 7D, the time resolution between two adjacent points is 40 milli-seconds. In another word, a second in Figures 7C and 7D is equivalent to 40-milli-second.

Figure 7A

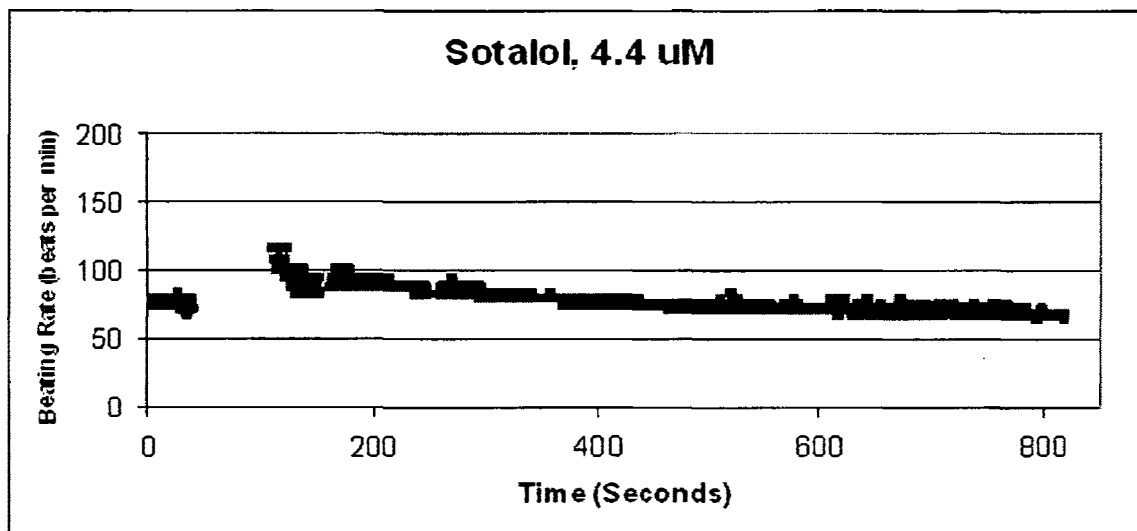


Figure 7B

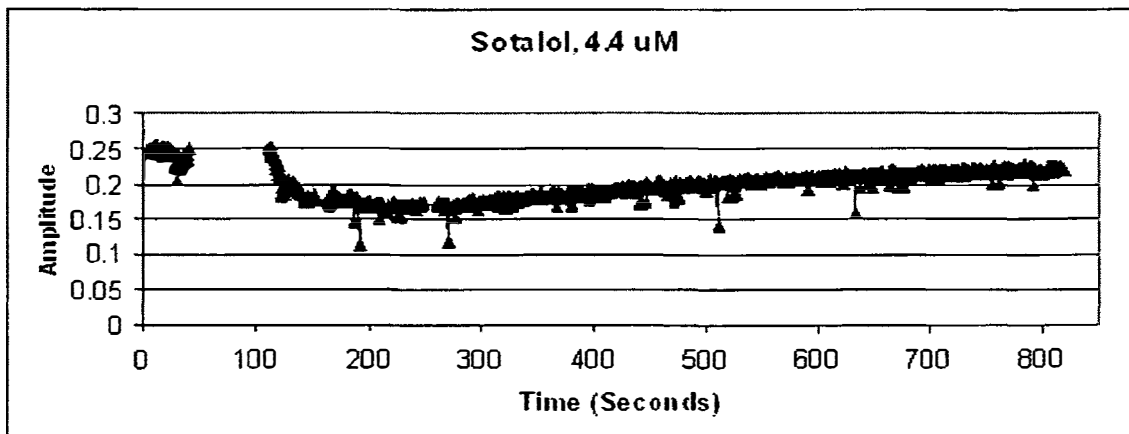


Figure 7C

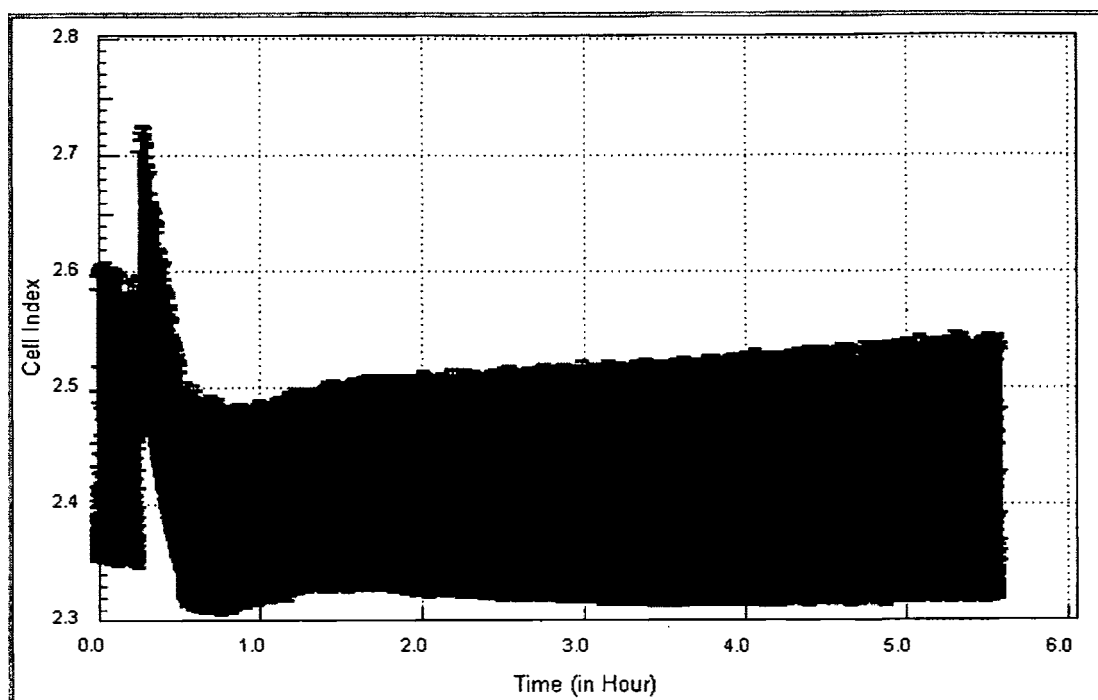


Figure 7D

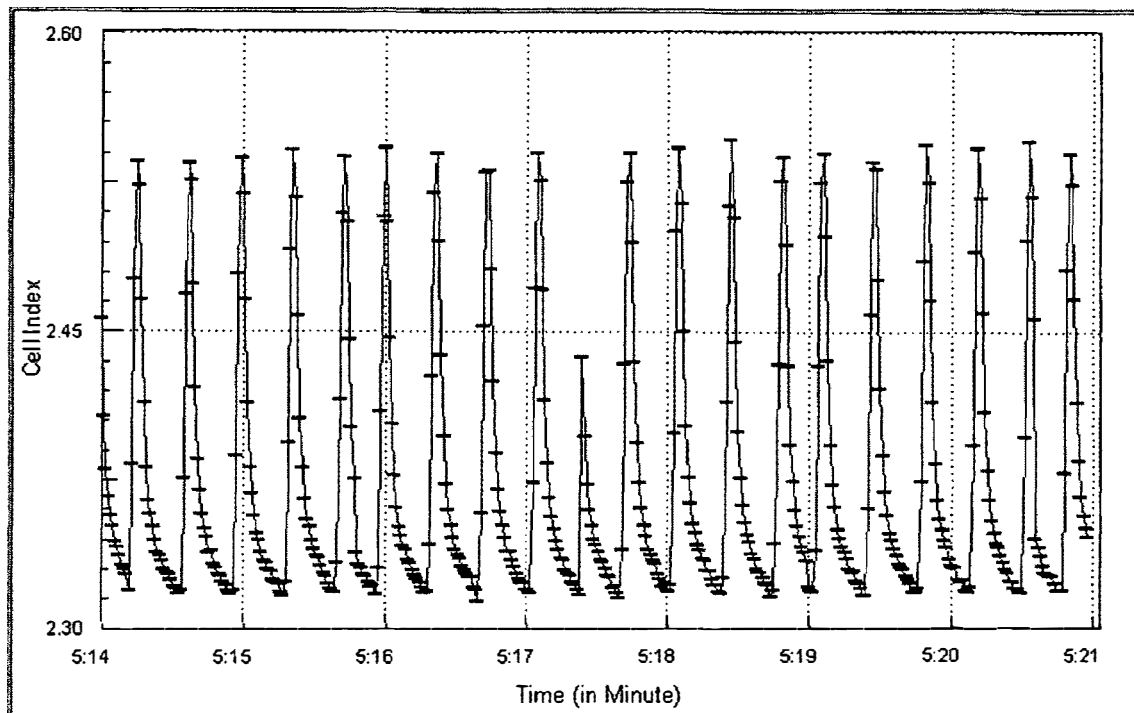


Figure 8A. Assessment of agents which slow down heart rate and cardiomyocyte beating

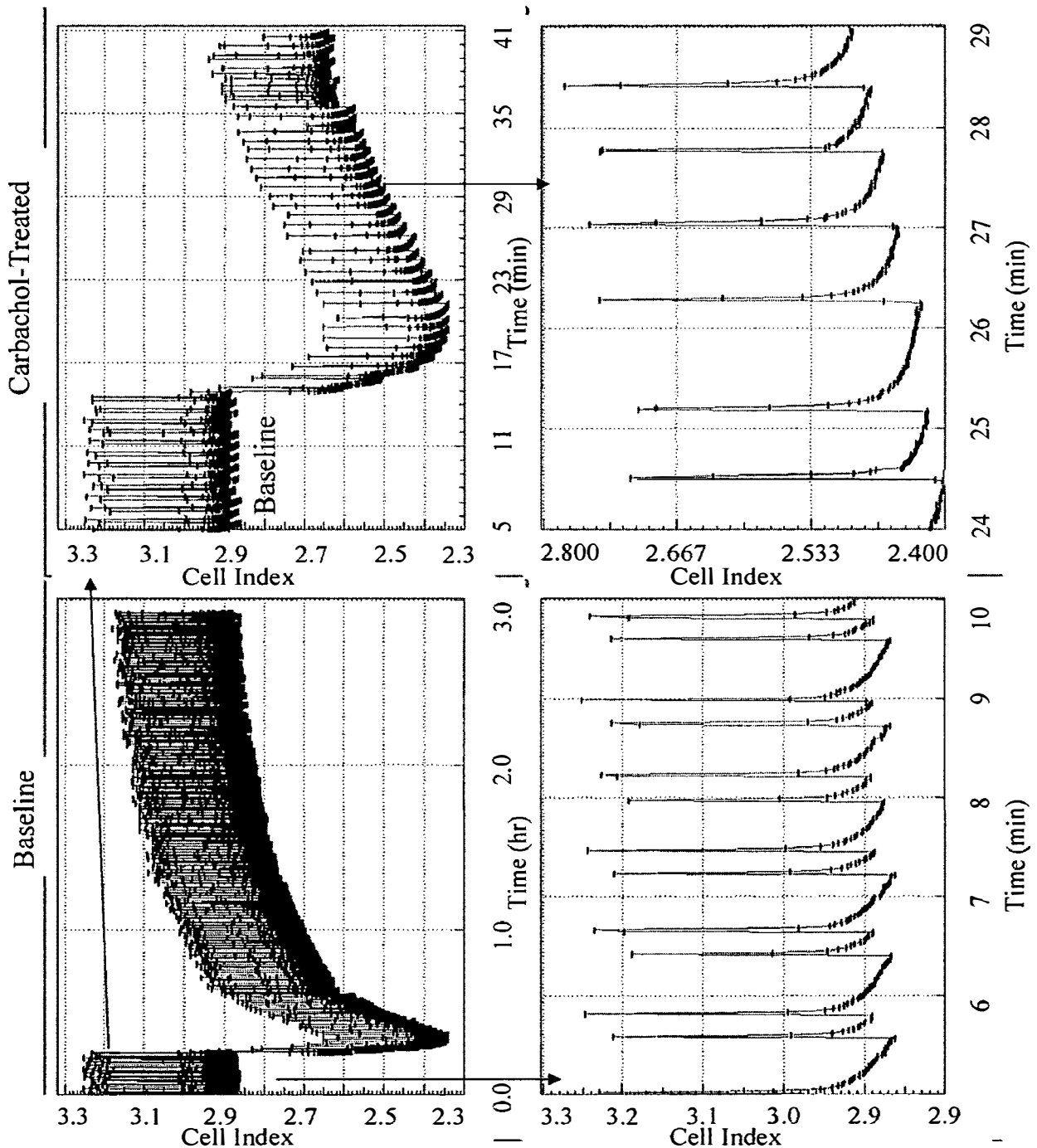


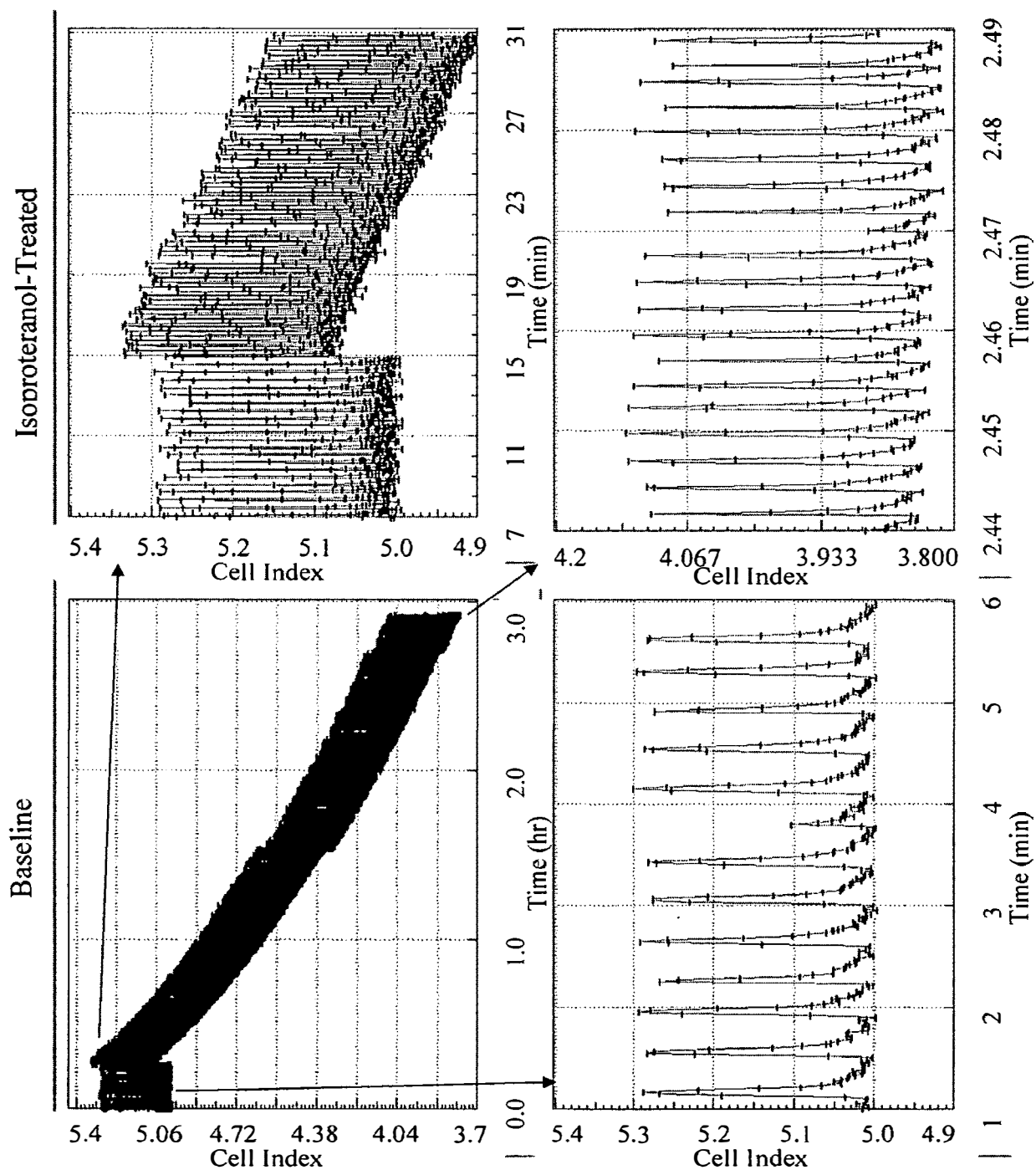
Figure 8B. Analysis of drugs which increase heart rate and cardiomyocyte beating

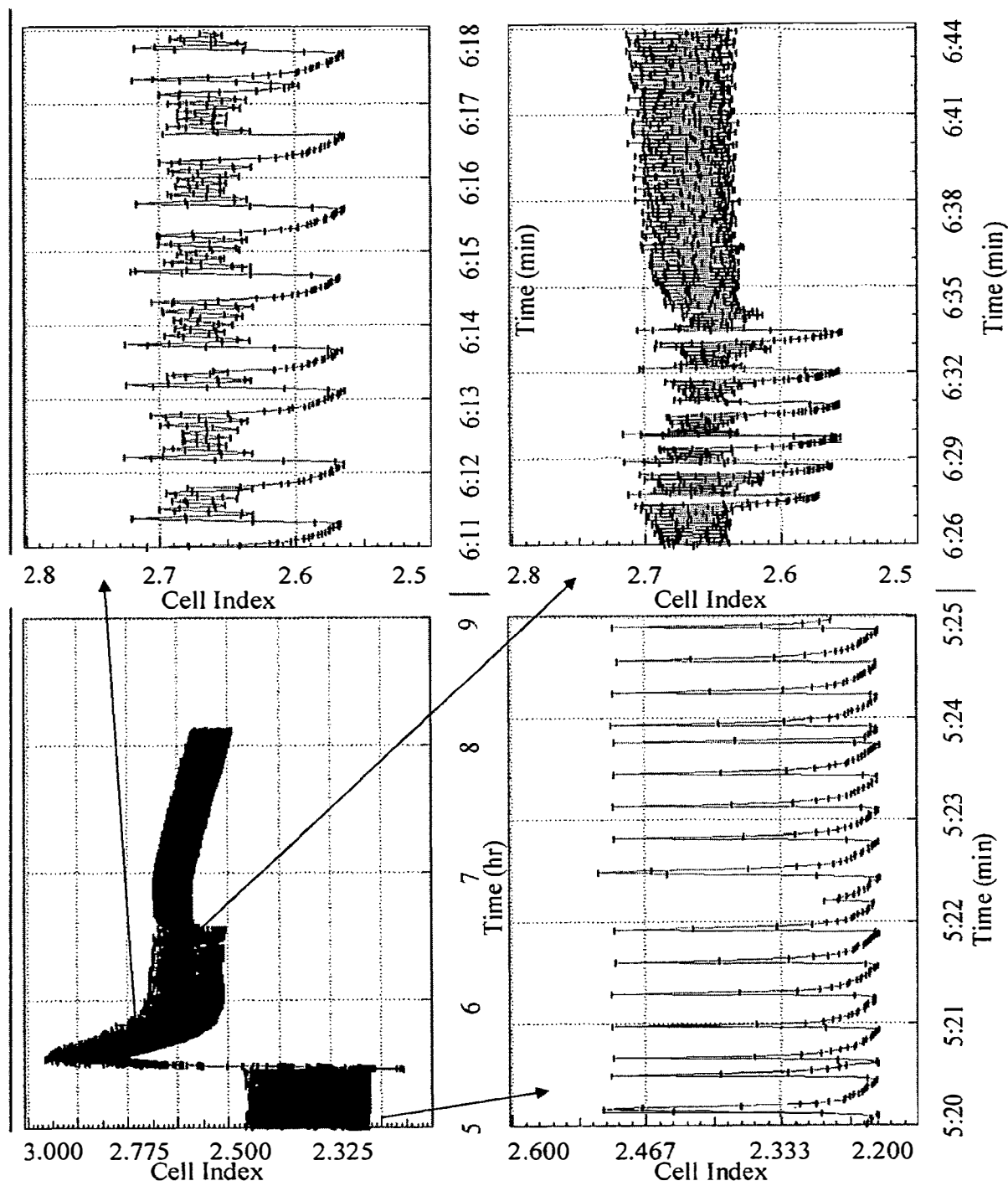
Figure 9A. Sotalol 33.3 μ M (0.033% DMSO)

Figure 9B. Astemizole 400 nM (0.004% DMSO)

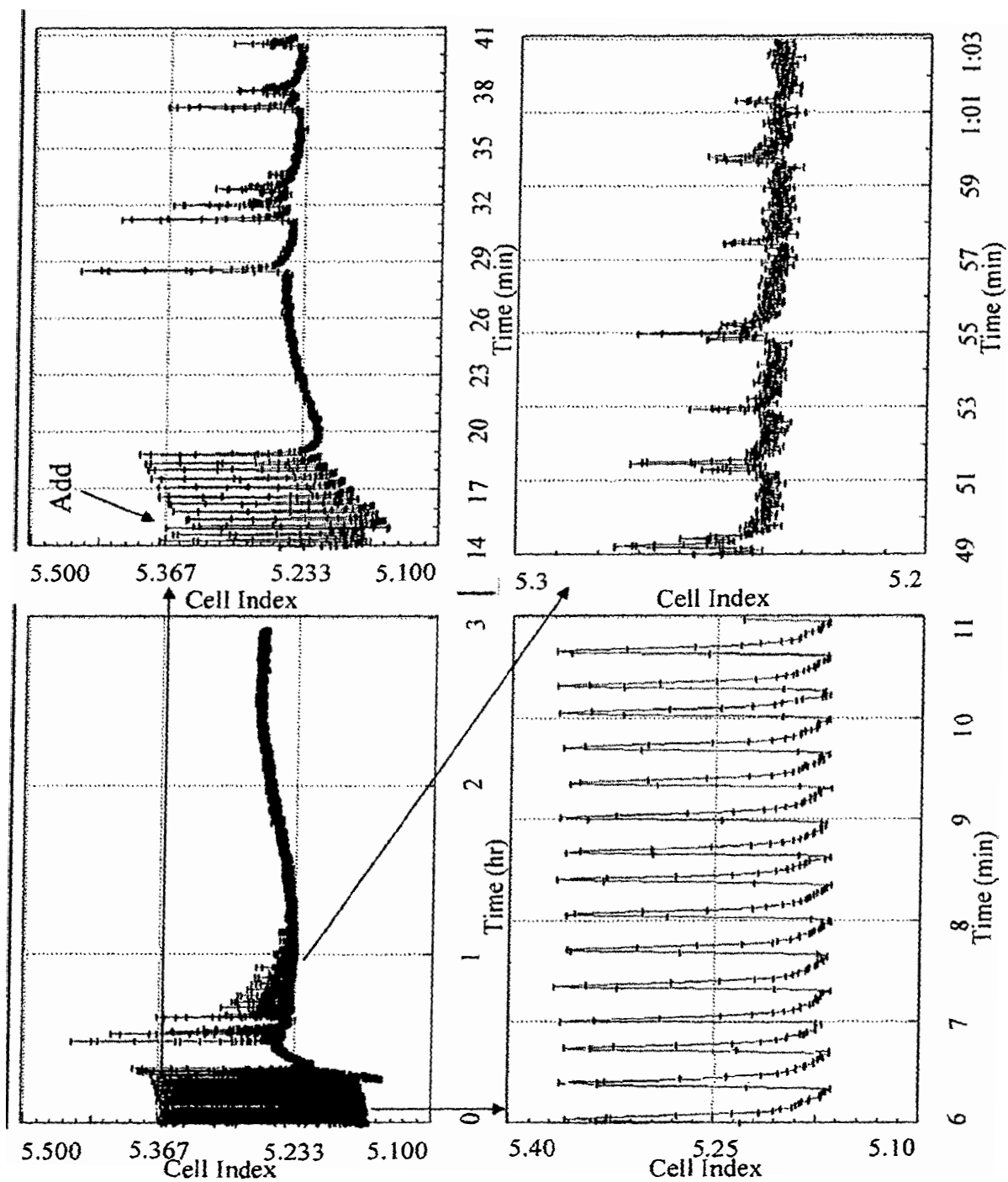


Figure 9C. Terfenadine 200 nM (0.065%DMSO)

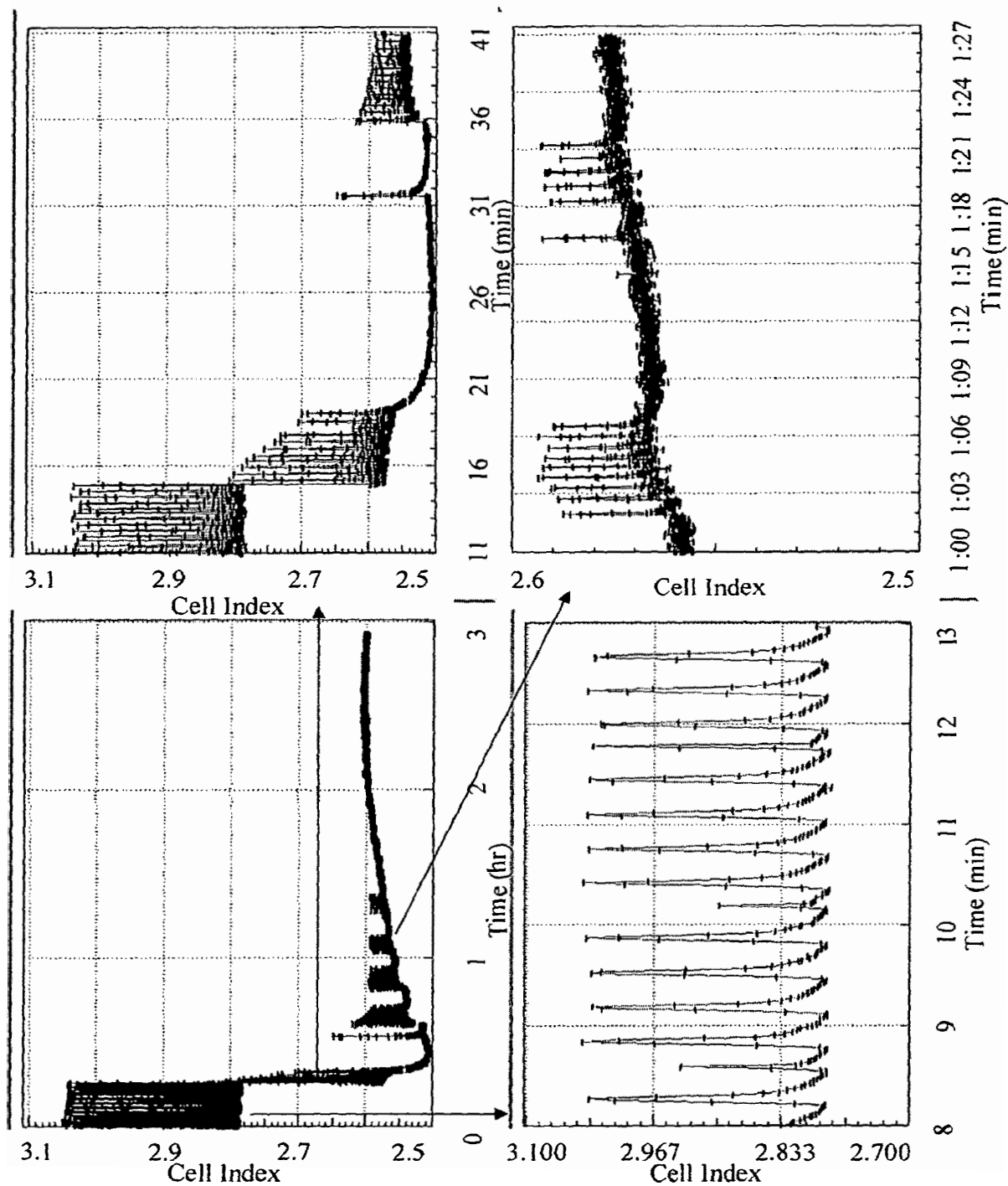


Figure 9D. Erythromycin 13.3 uM (0.13% DMSO)

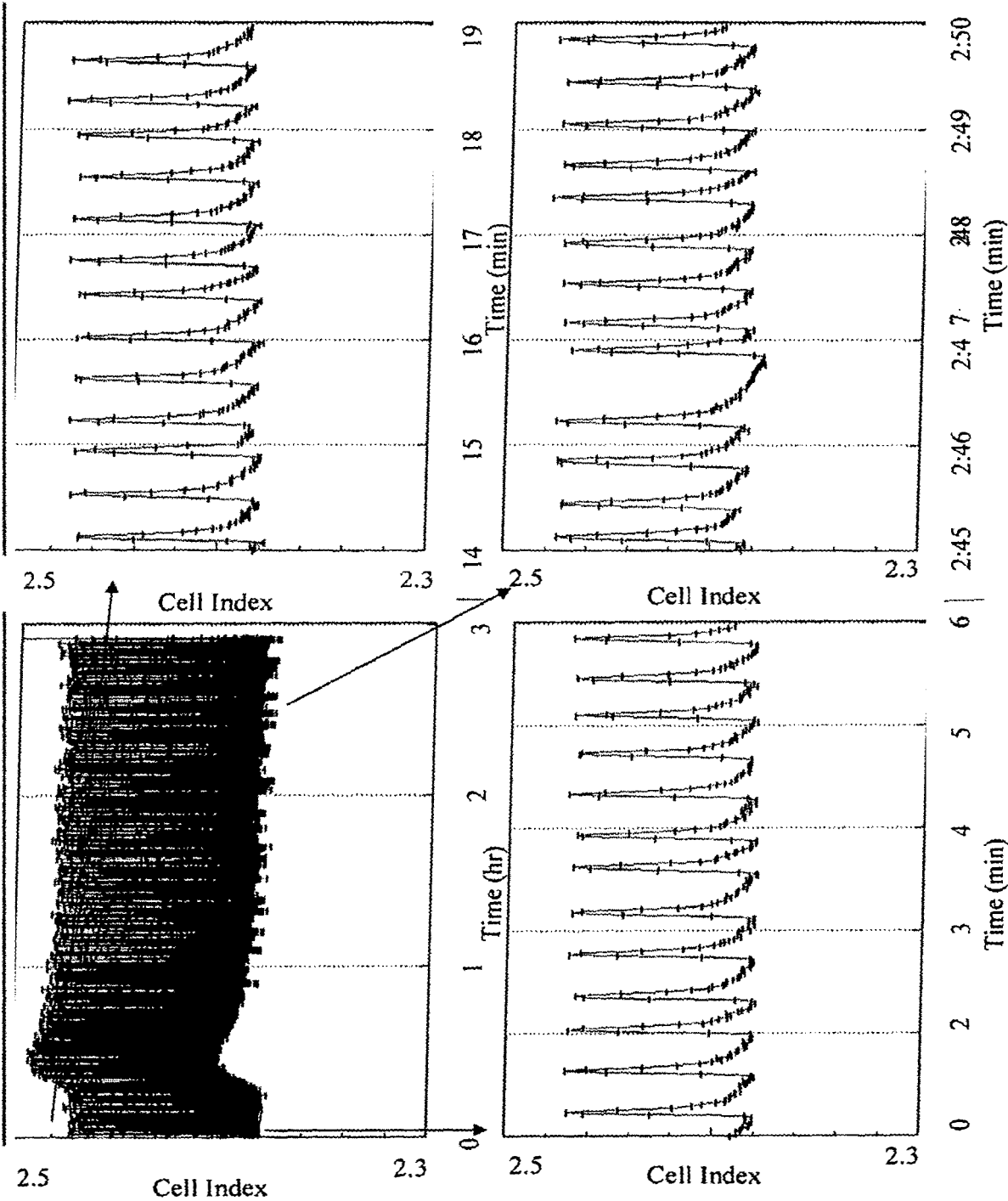


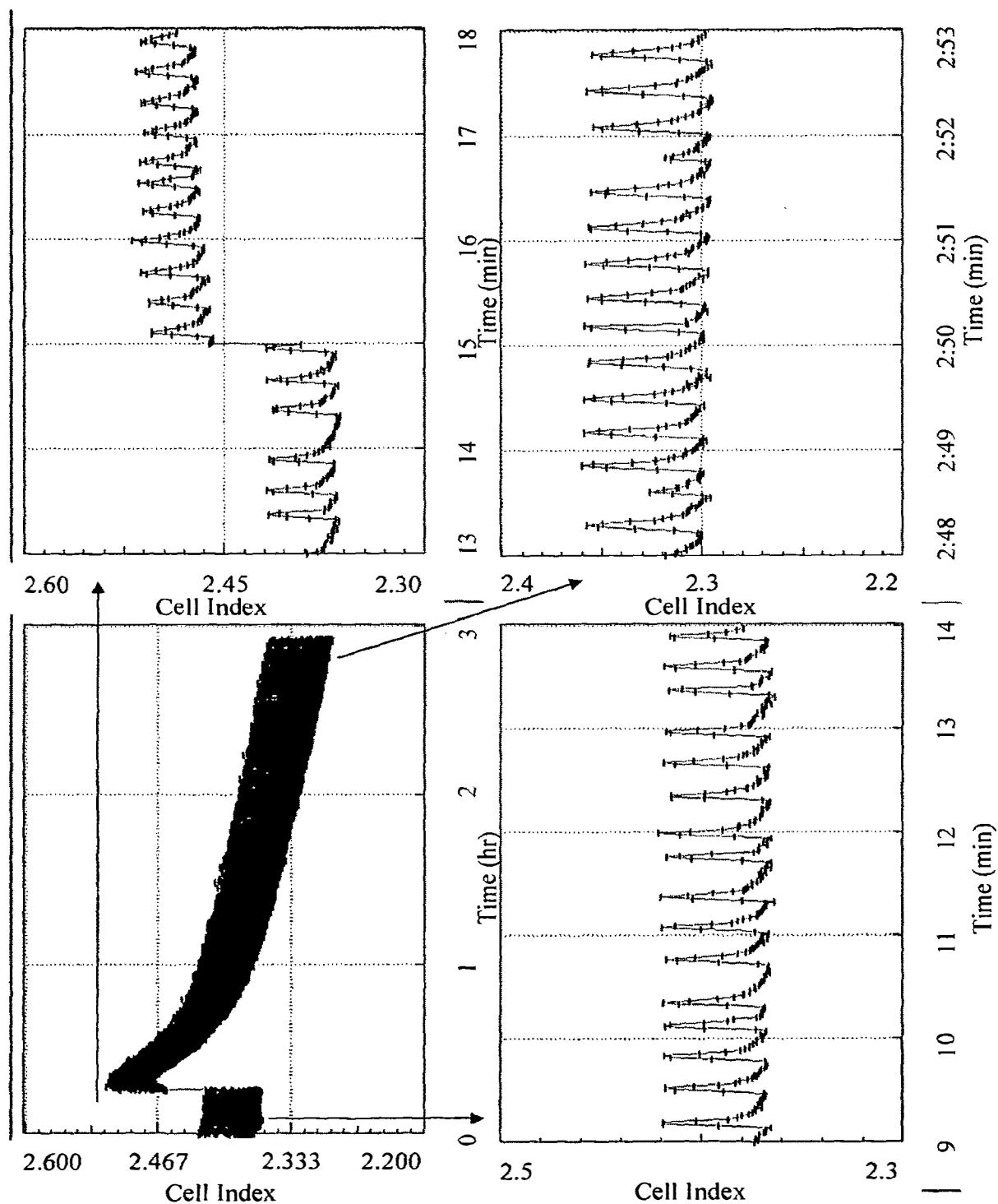
Figure 9E. Moxifloxacin 20 μ M (0.3% DMSO)

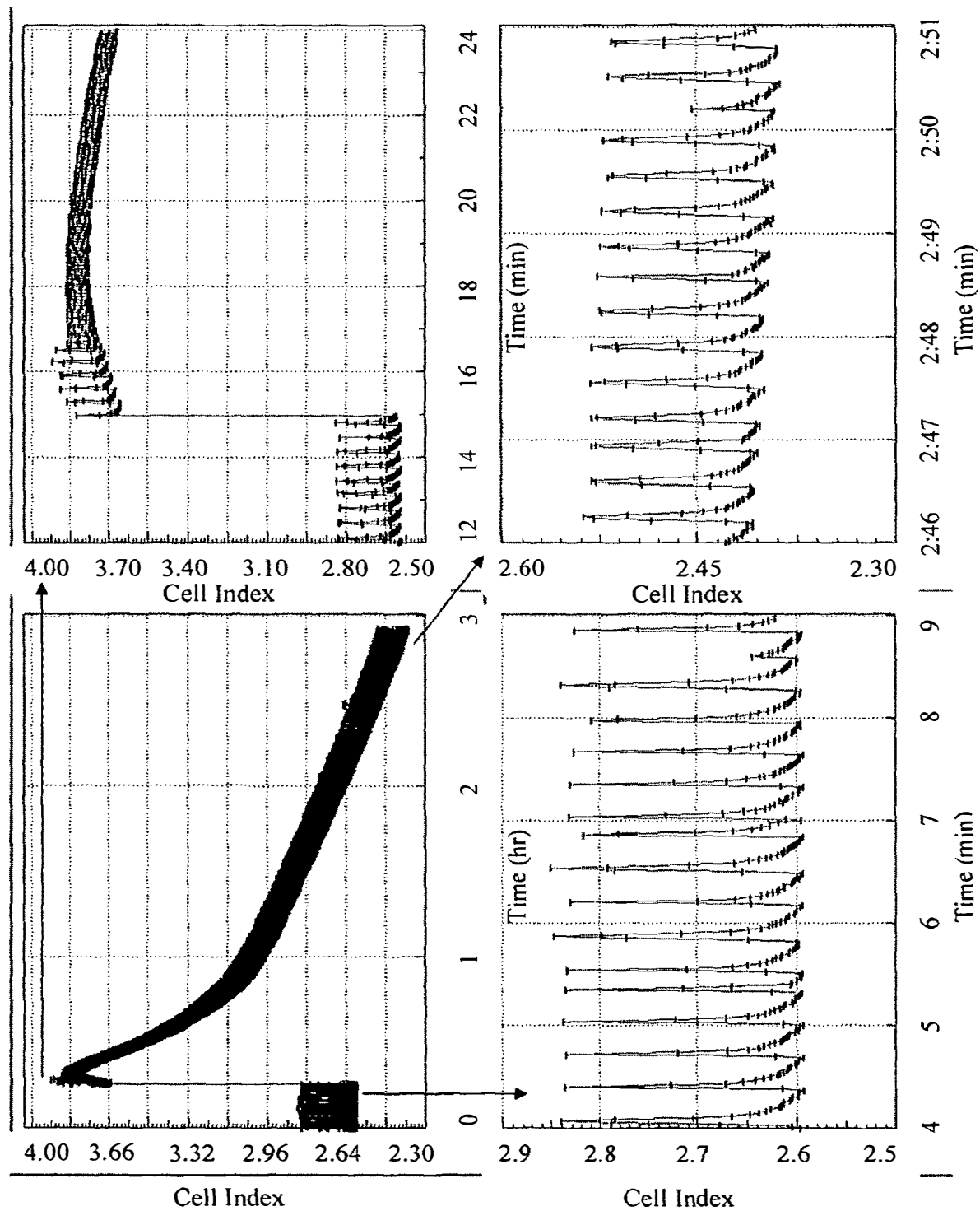
Figure 9F. Pentamidine 20uM (0.2% DMSO)

Figure 9G. Amitriptyline 4.4uM(0.04 % DMSO)

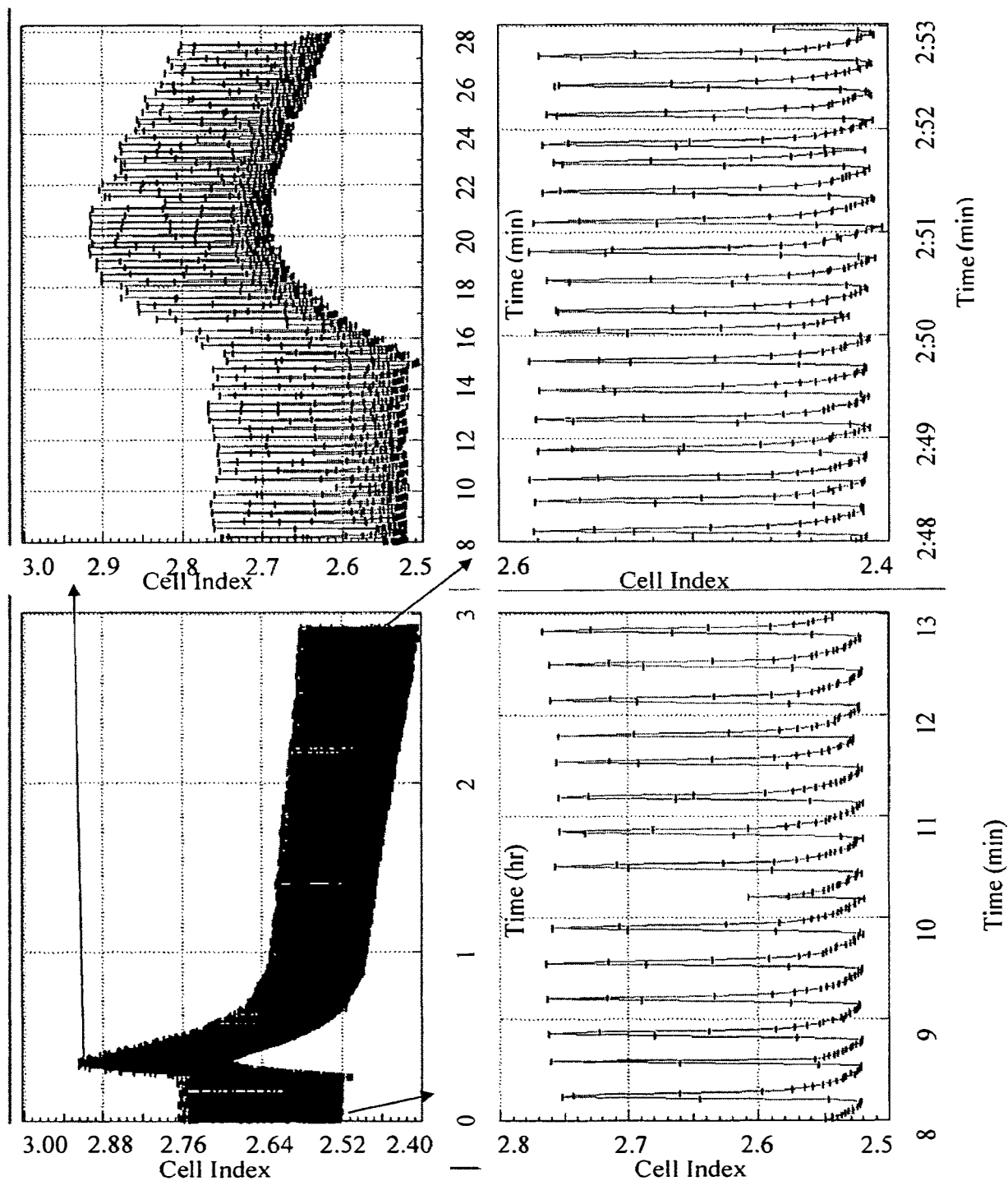


Figure 9H. Verapamil 130 nM (0.0013% DMSO)

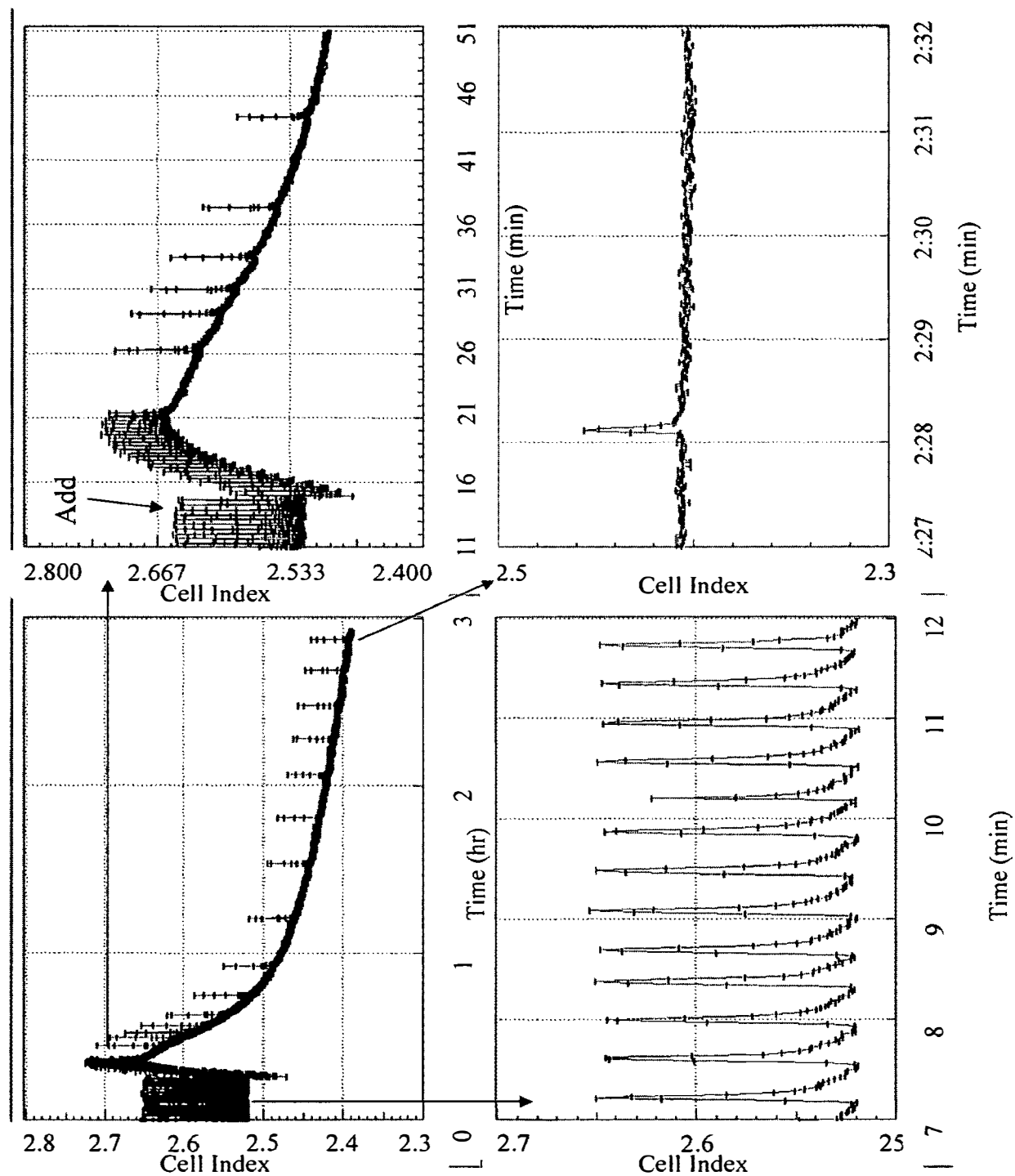


Figure 9I. Rosiglitazone 13.3 uM (0.13% DMSO)

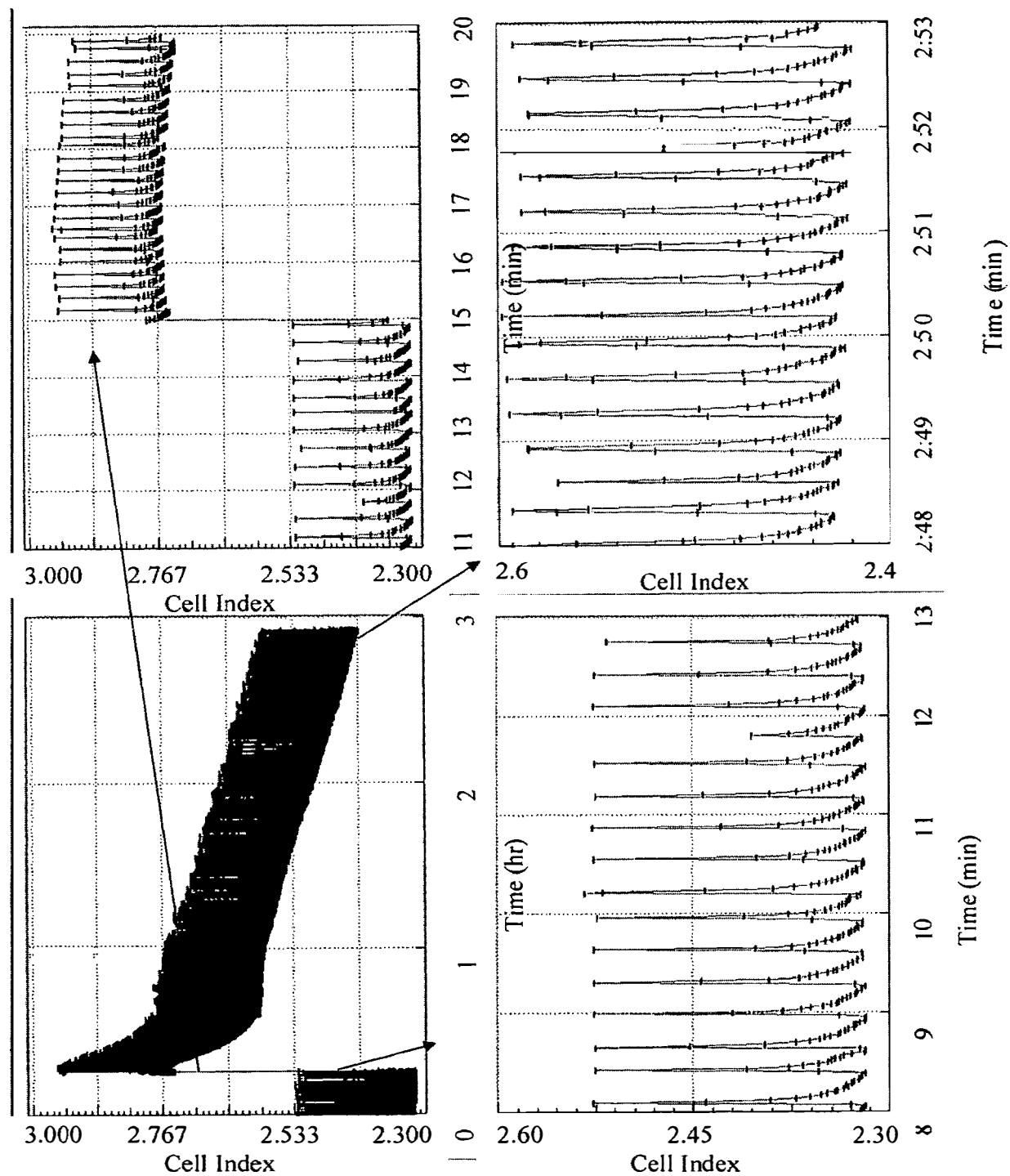


Figure 9J. Rofecoxib 13.3 uM

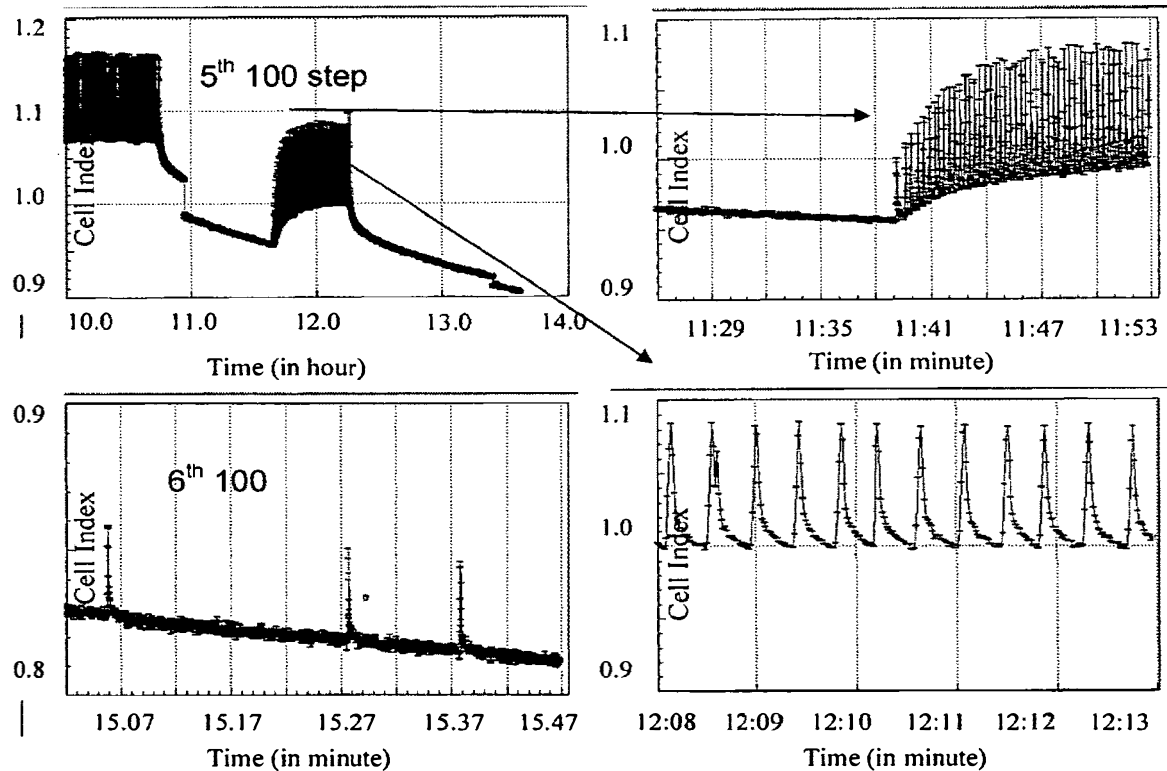
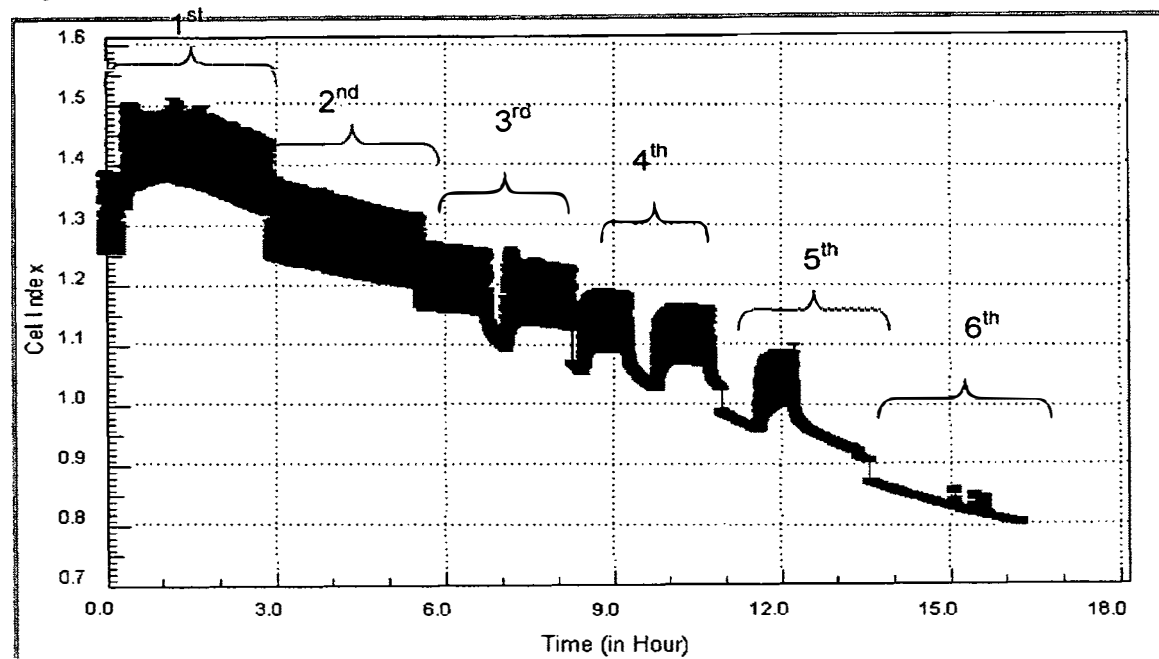


Figure 9K. Celecoxib 4.4 uM

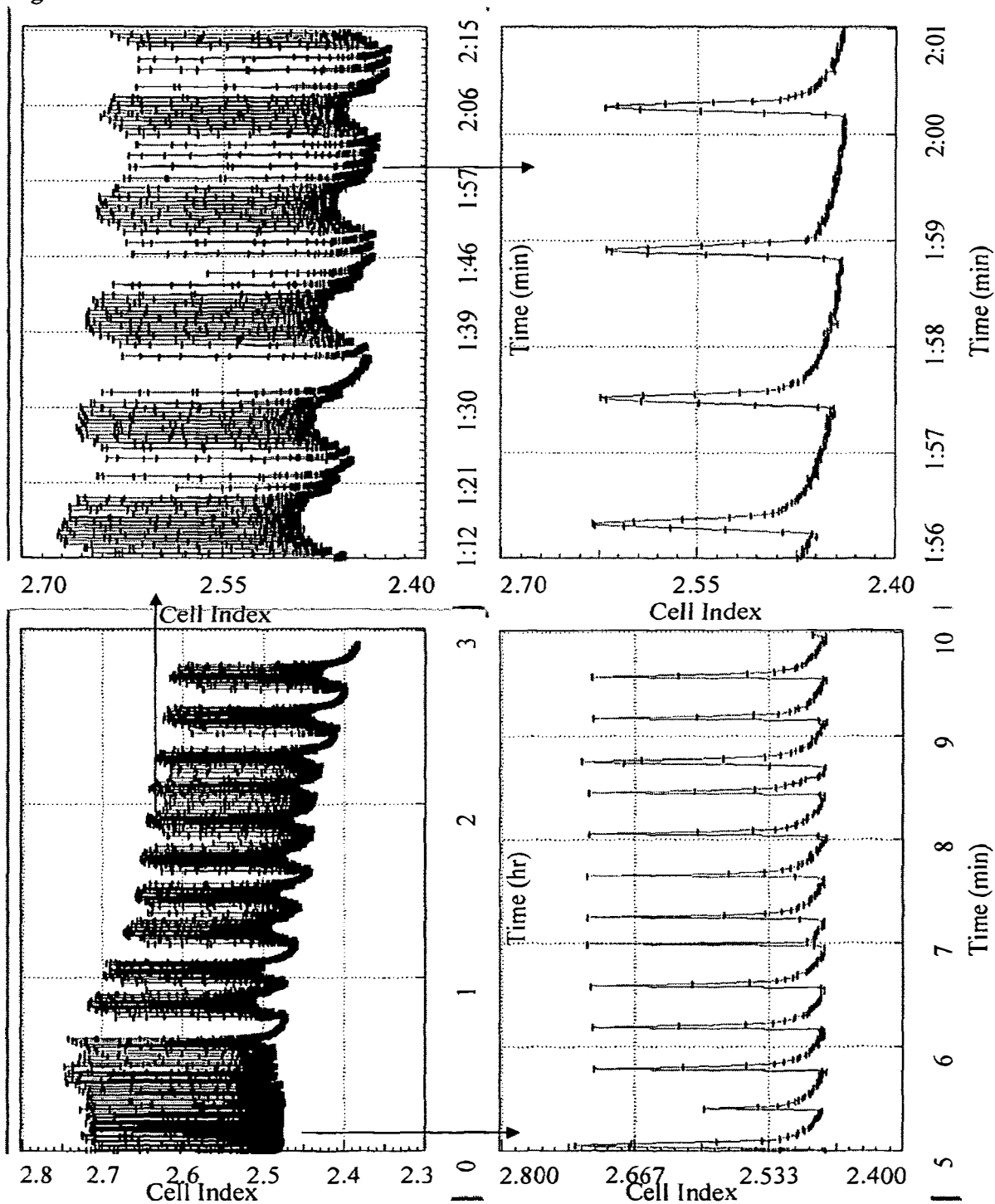


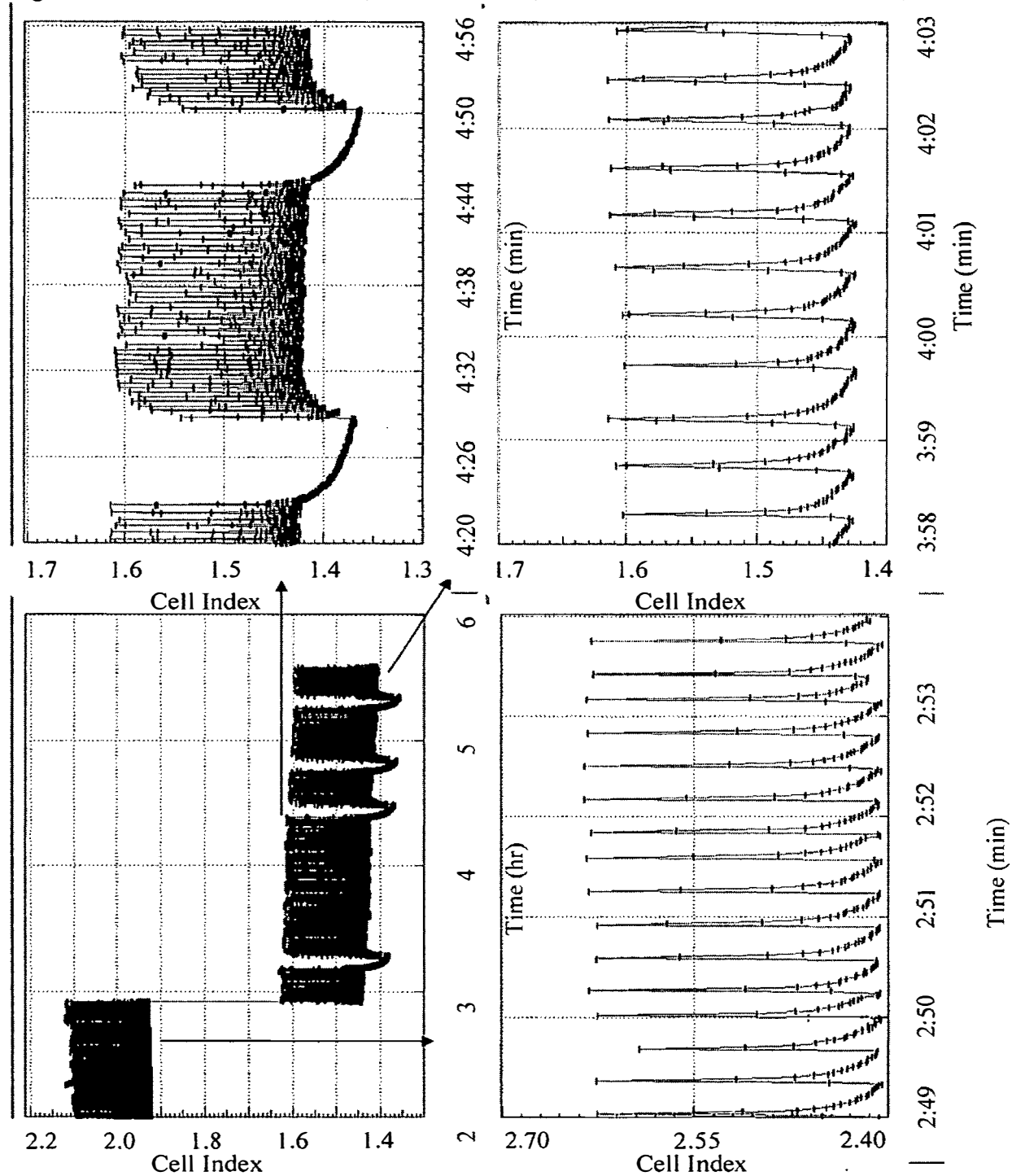
Figure 9L. Doxorubicin 40 μ M (0.4% DMSO) (measurement after 2h treatment)

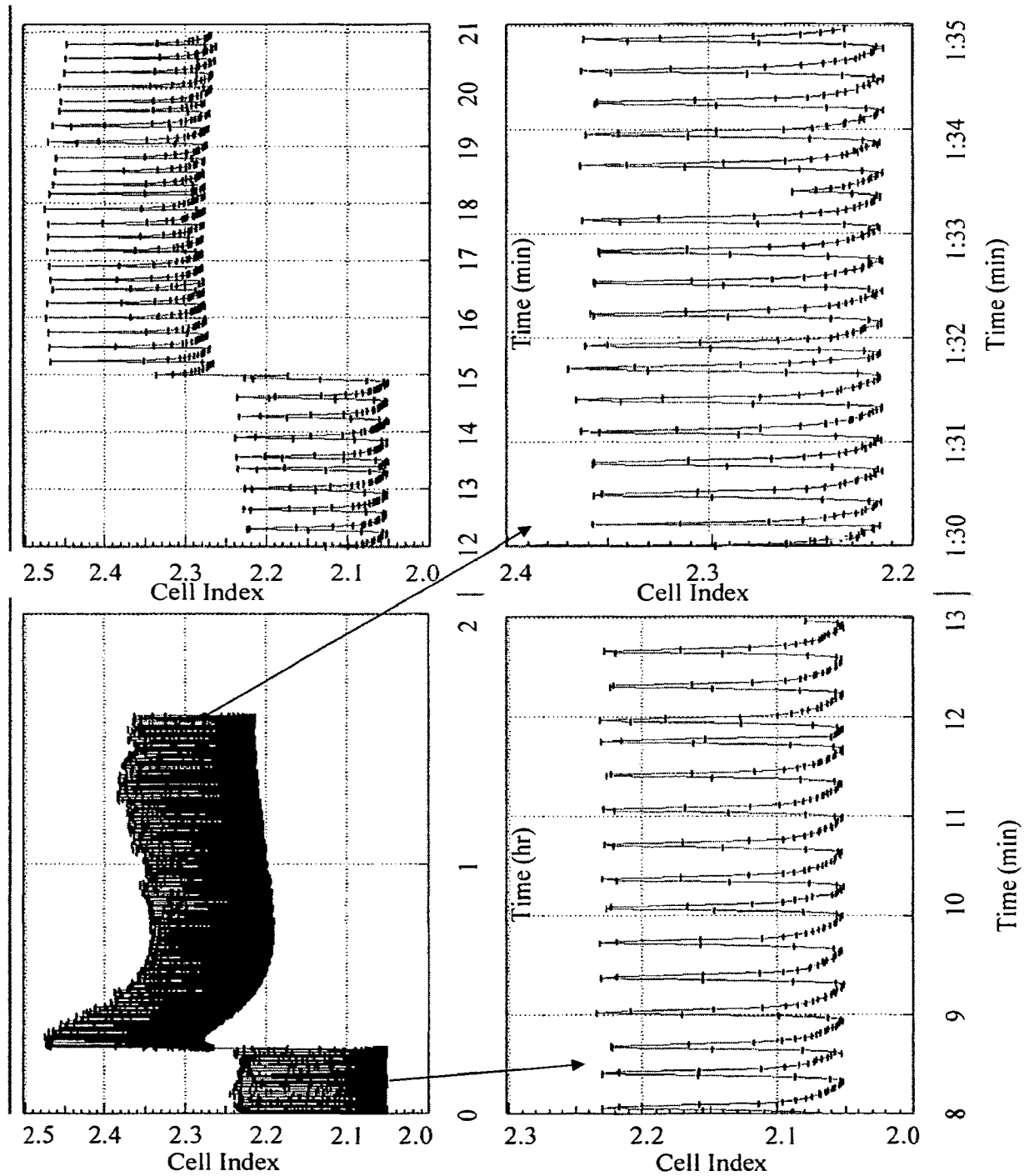
Figure 9M. Cyclosporine A 13.3uM (0.13% DMSO)

Figure 9N. Propranolol 4.4uM (0.04% DMSO)

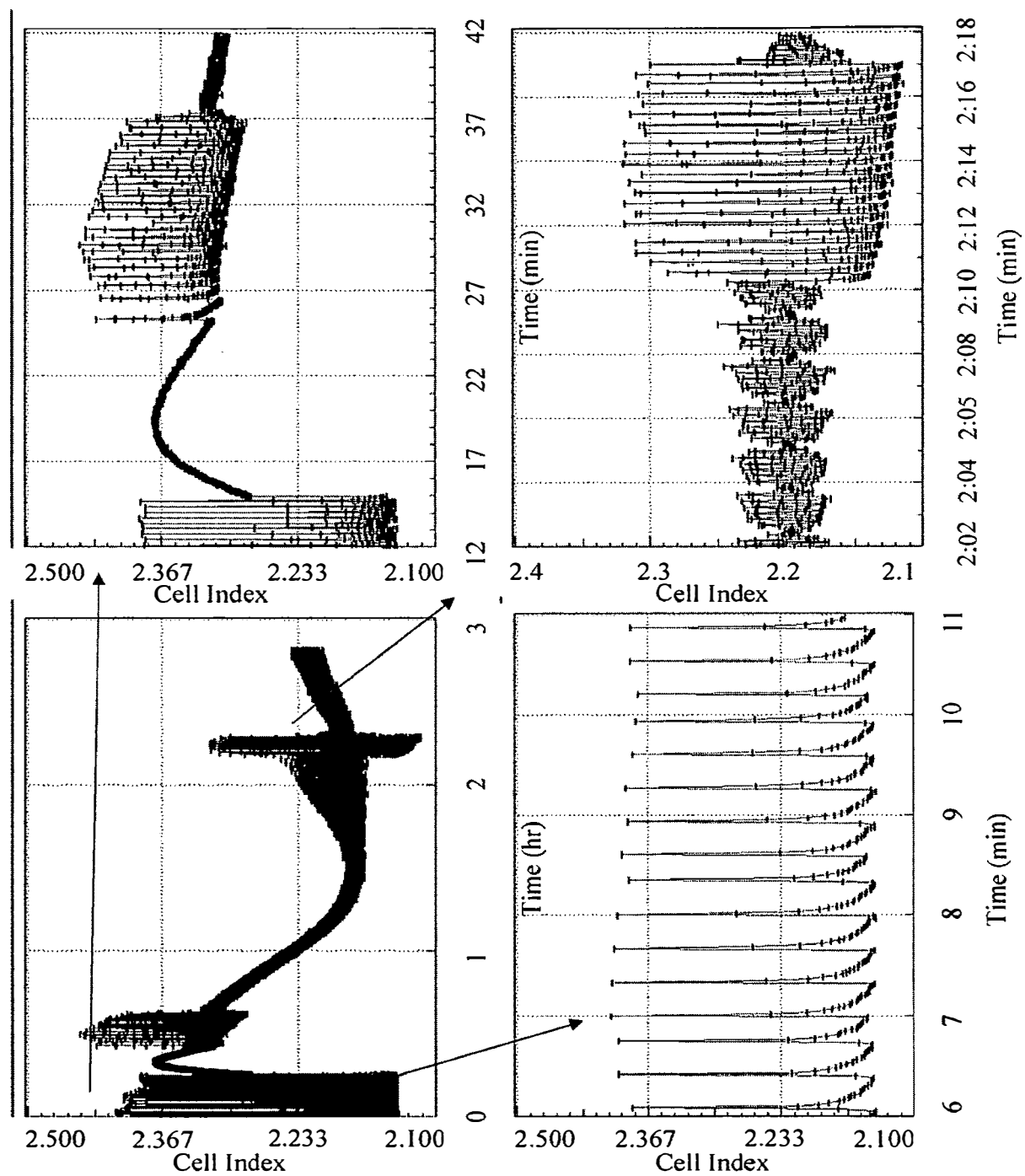


Figure 9O. E4031 9.1 nM

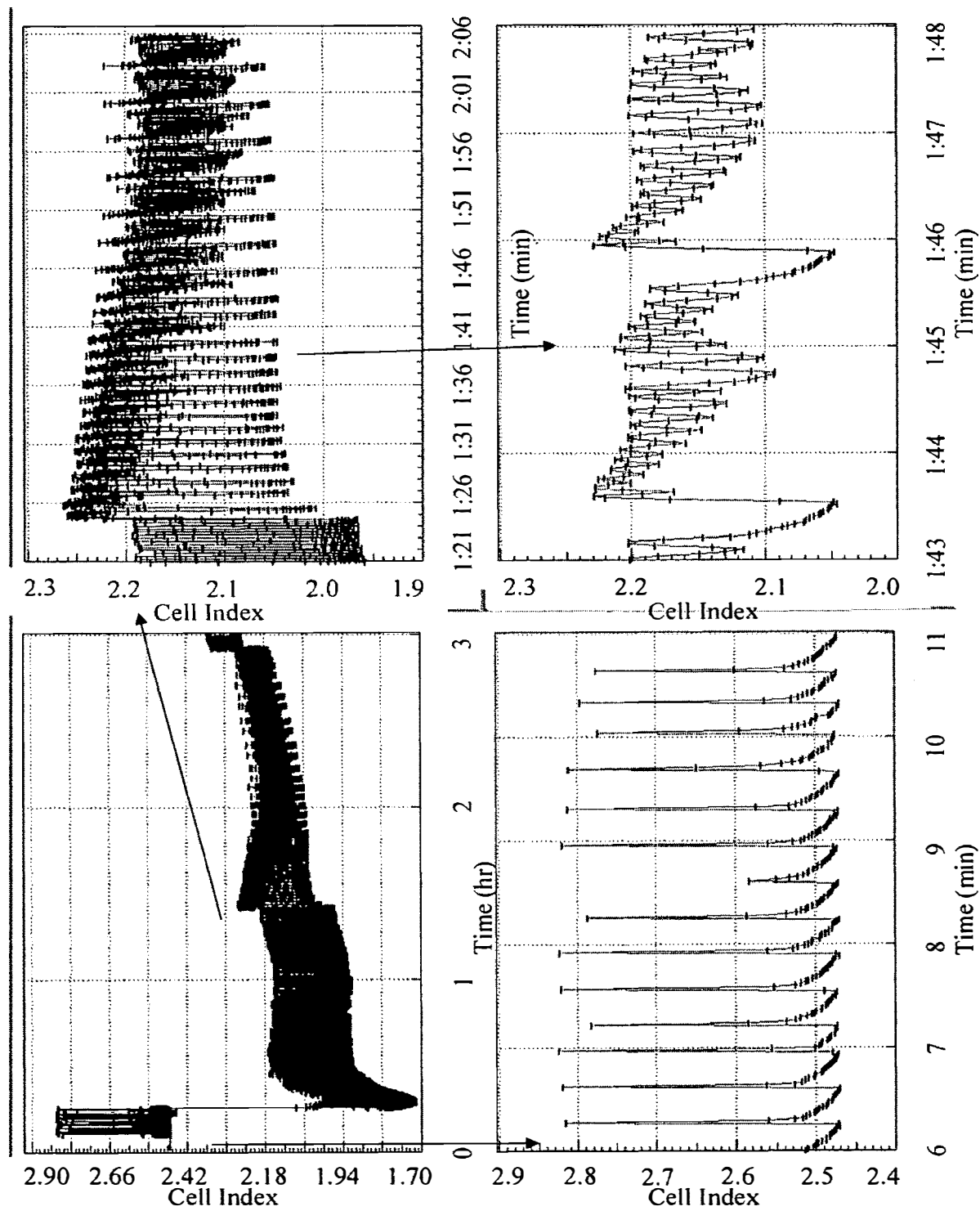


Figure 9P. DDT 8uM (0.08% DMSO)

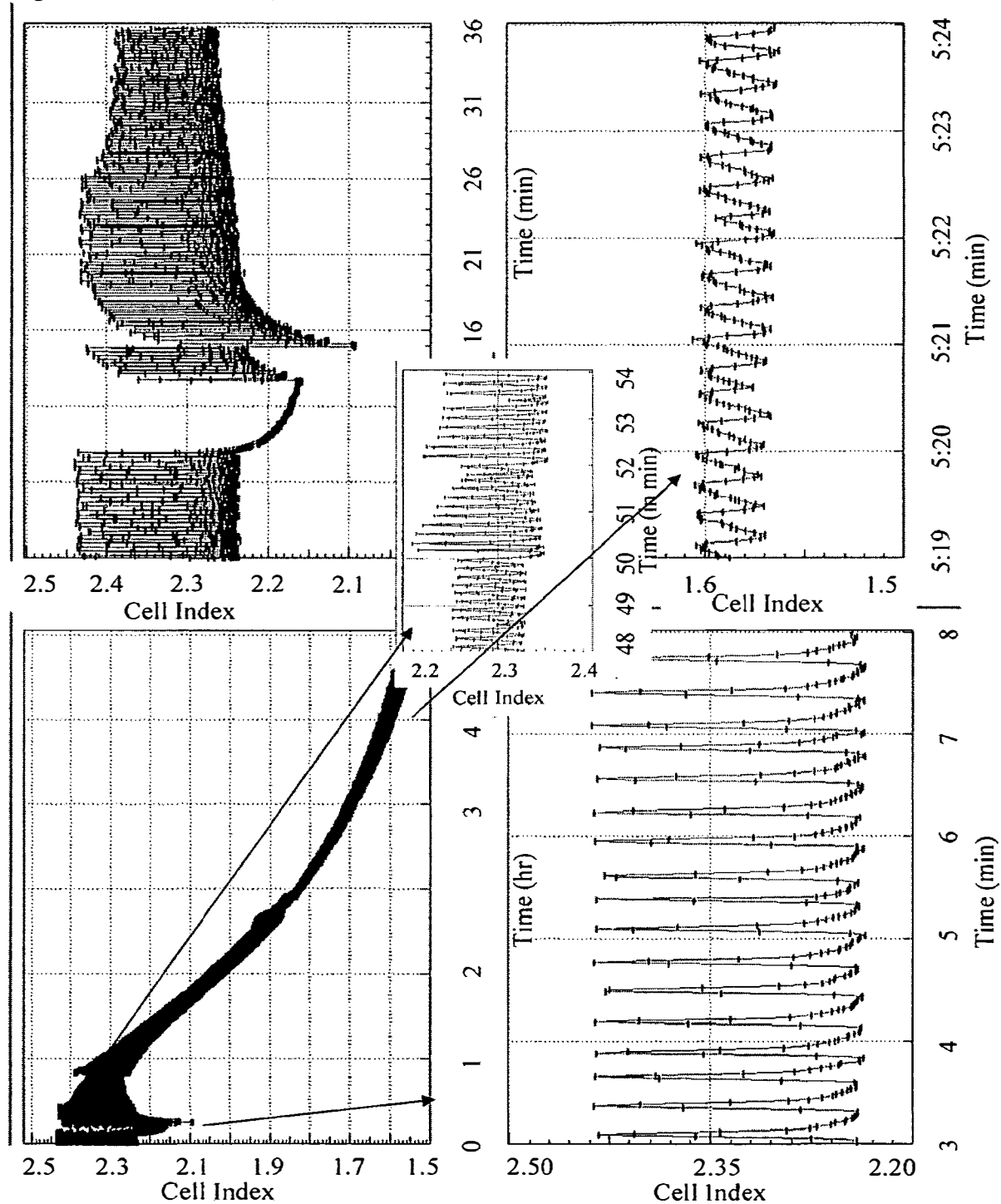


Figure 9Q. PCB 8uM (DMSO 0.08%)

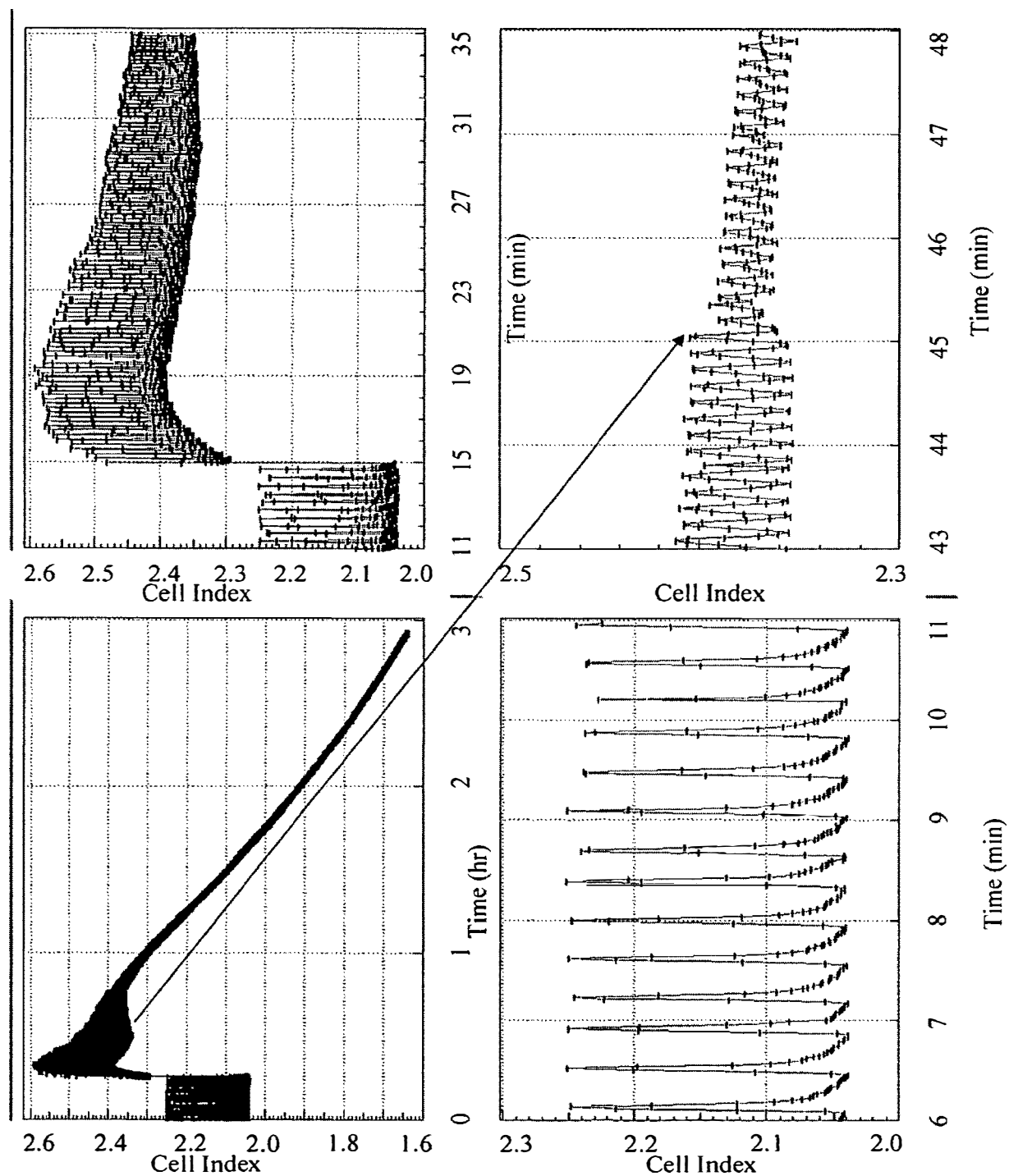


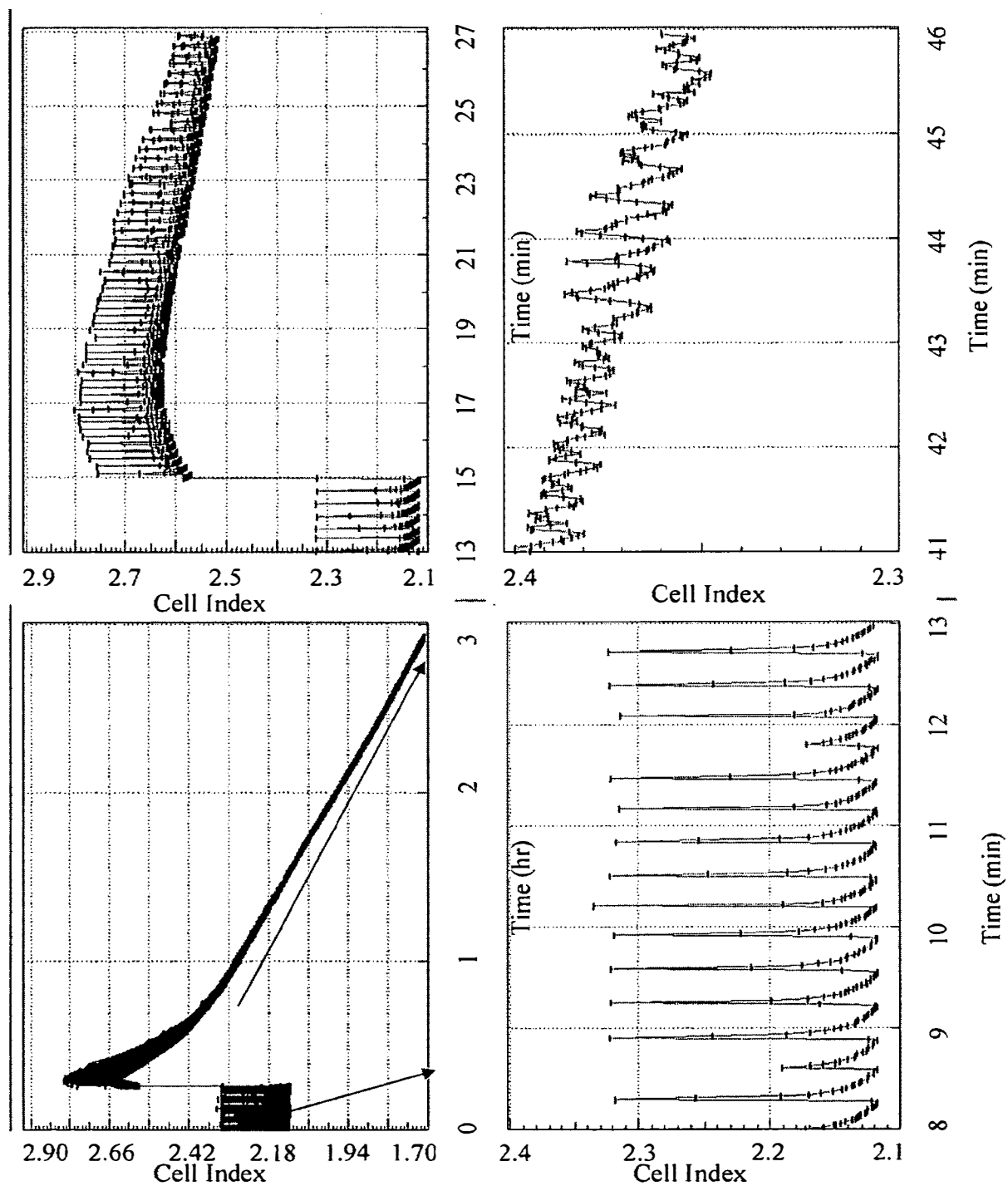
Figure 9R. Endosulfan 8uM (0.08% DMSO)

Figure 9S. DMSO 0.13%

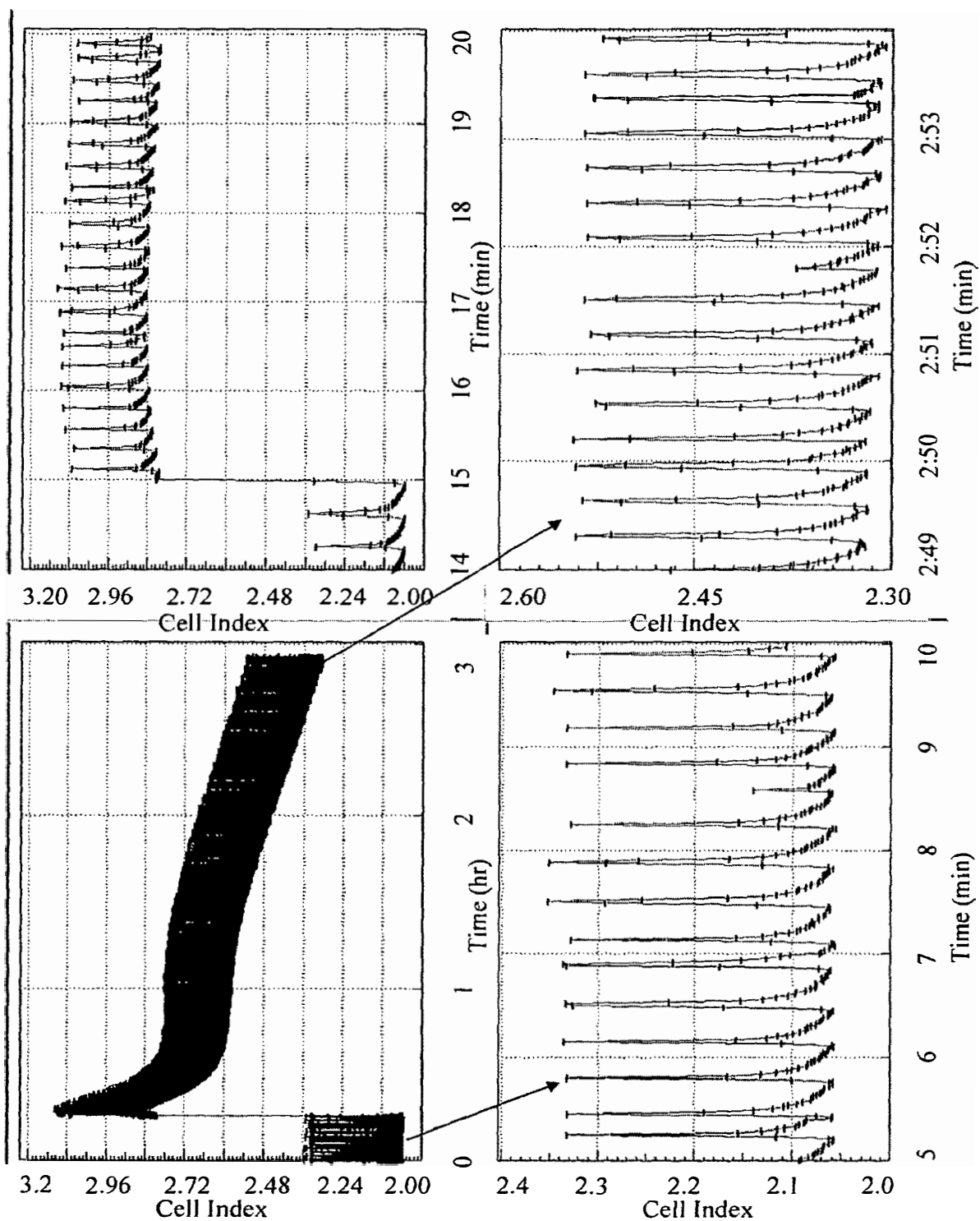


Figure 10. Cardiotoxic Patterns

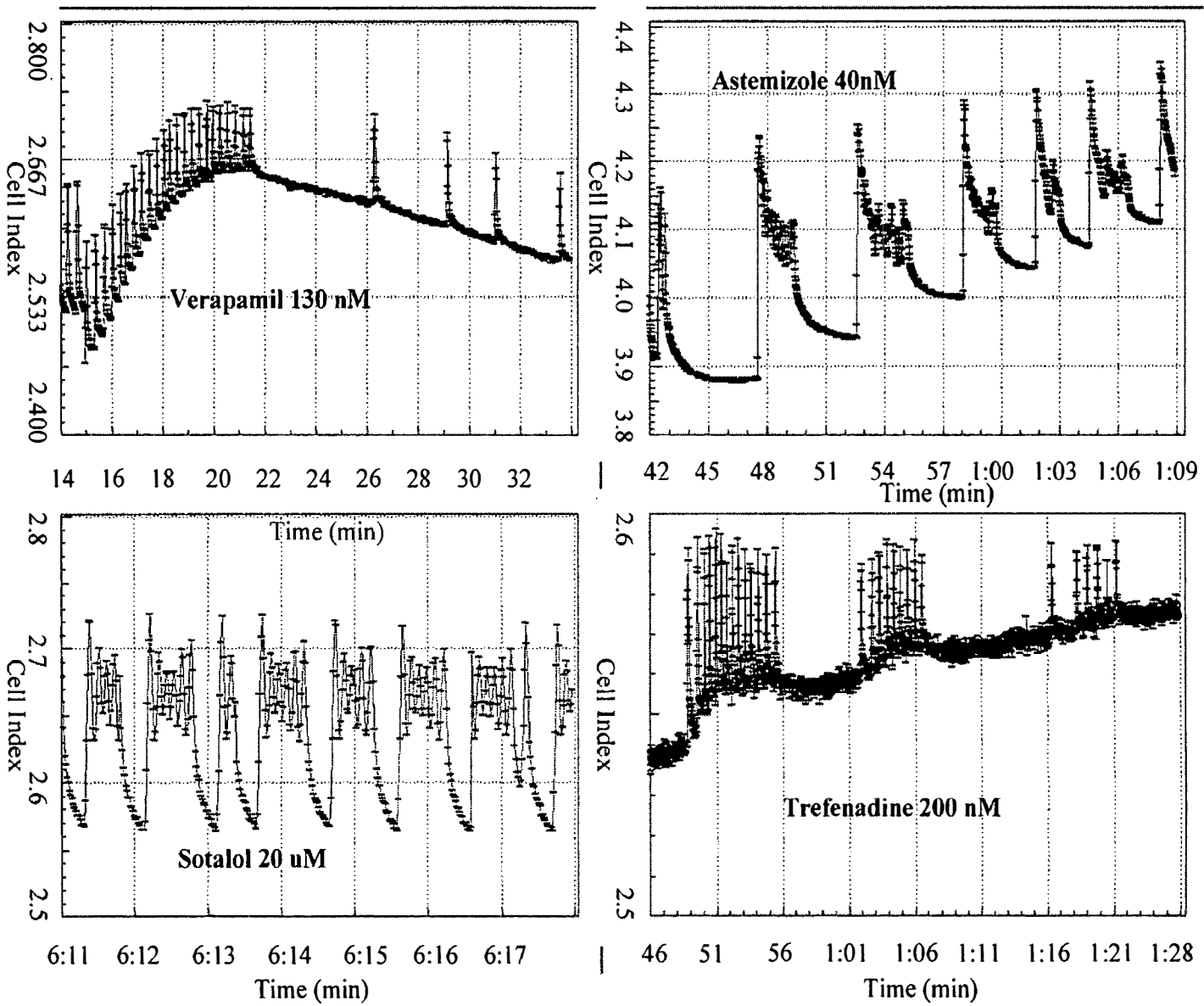


Figure 10. Cardiotoxic Patterns (continued)

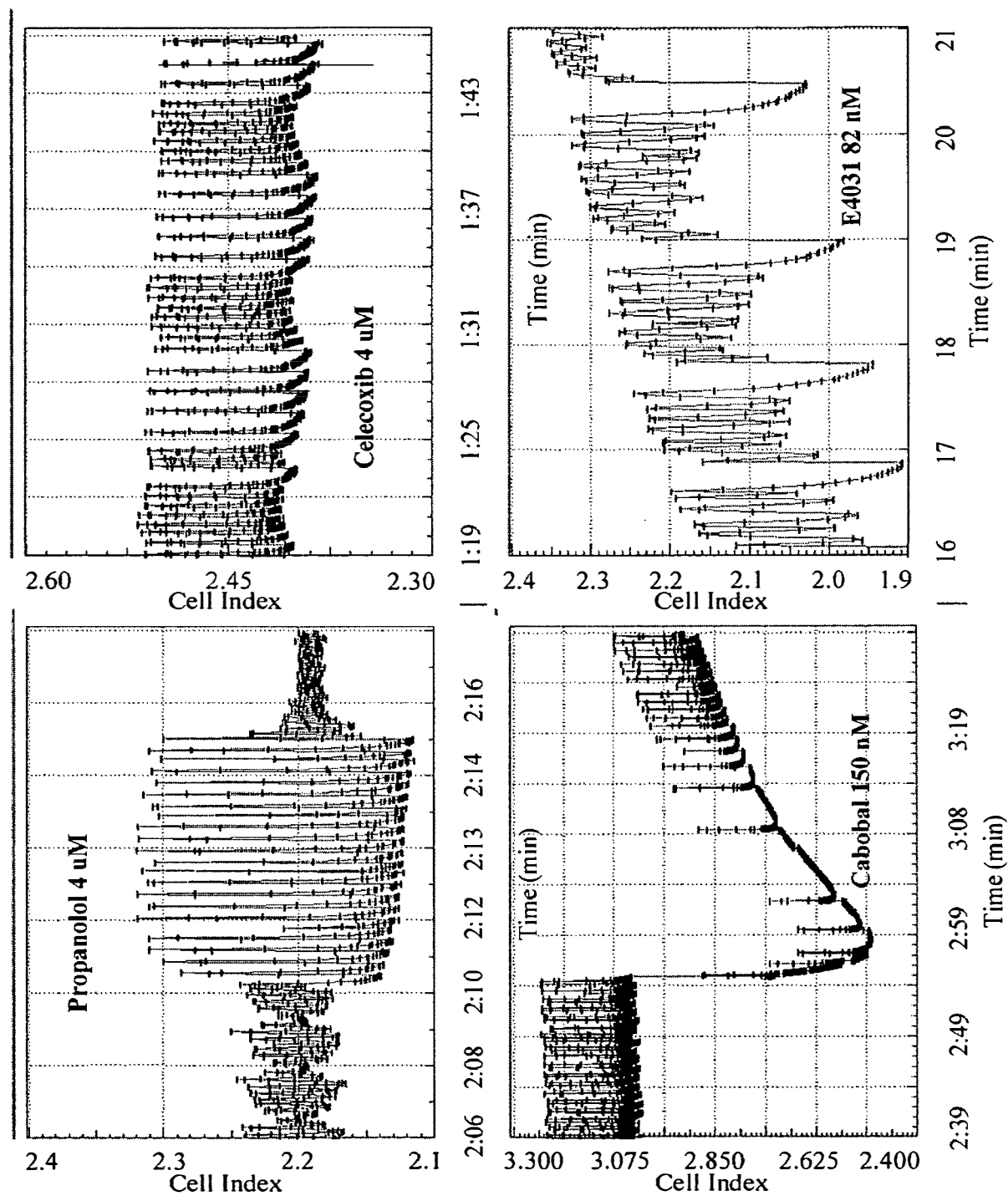


Figure 11.

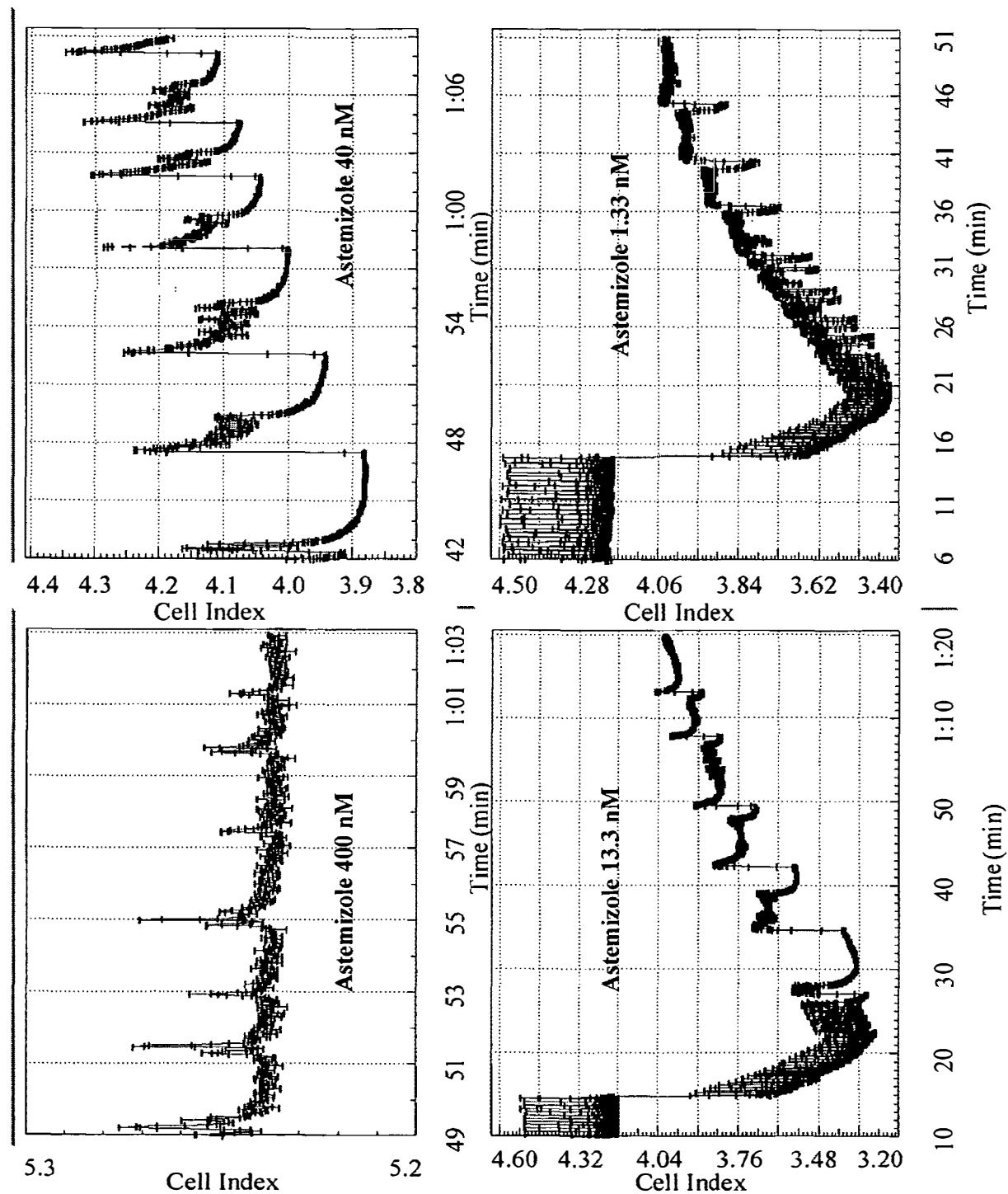
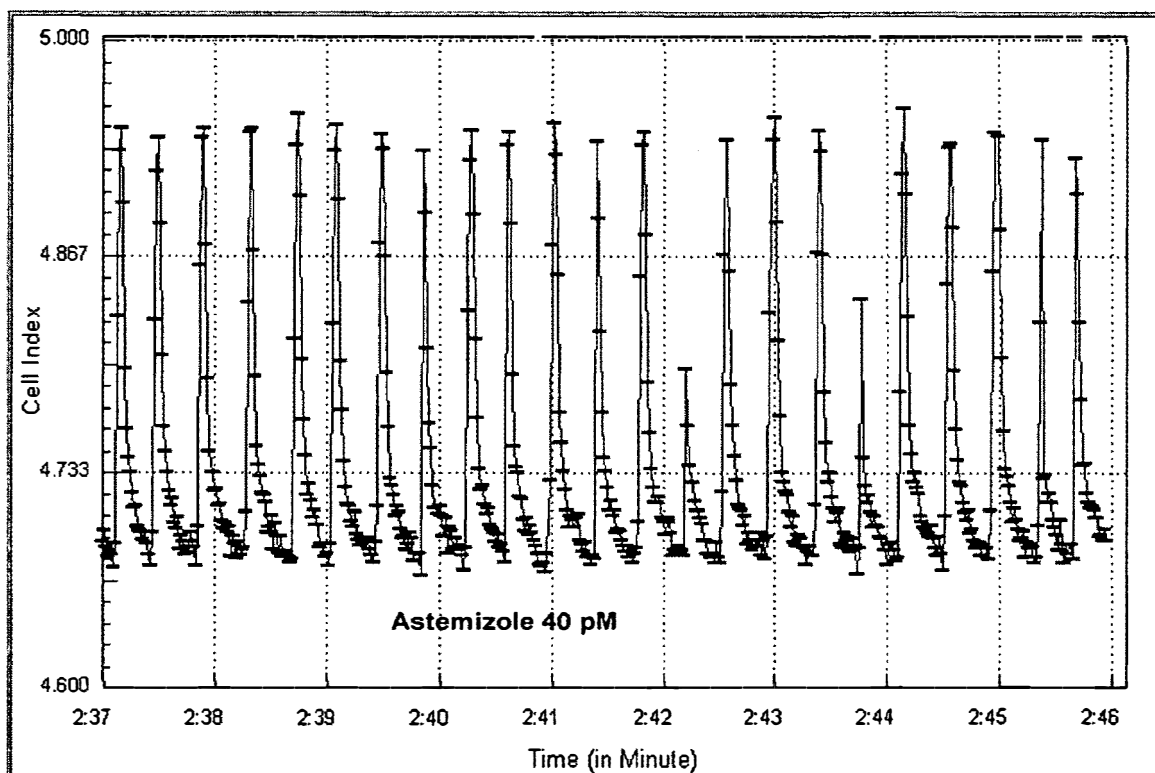
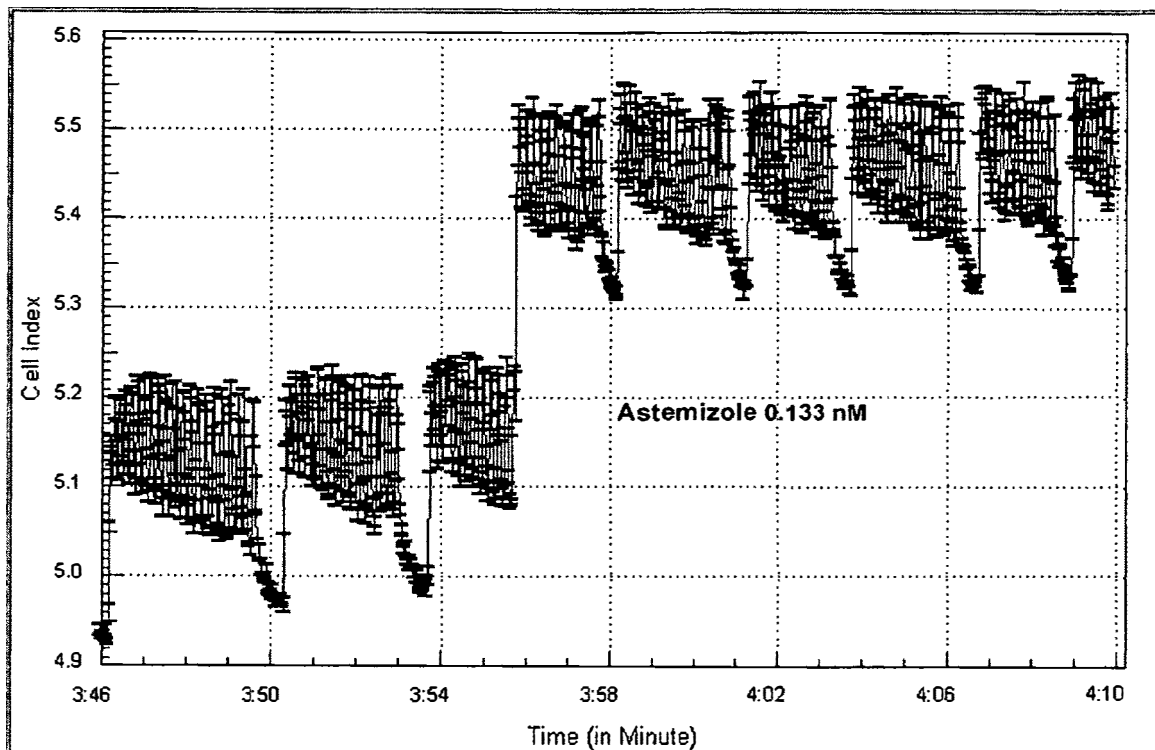
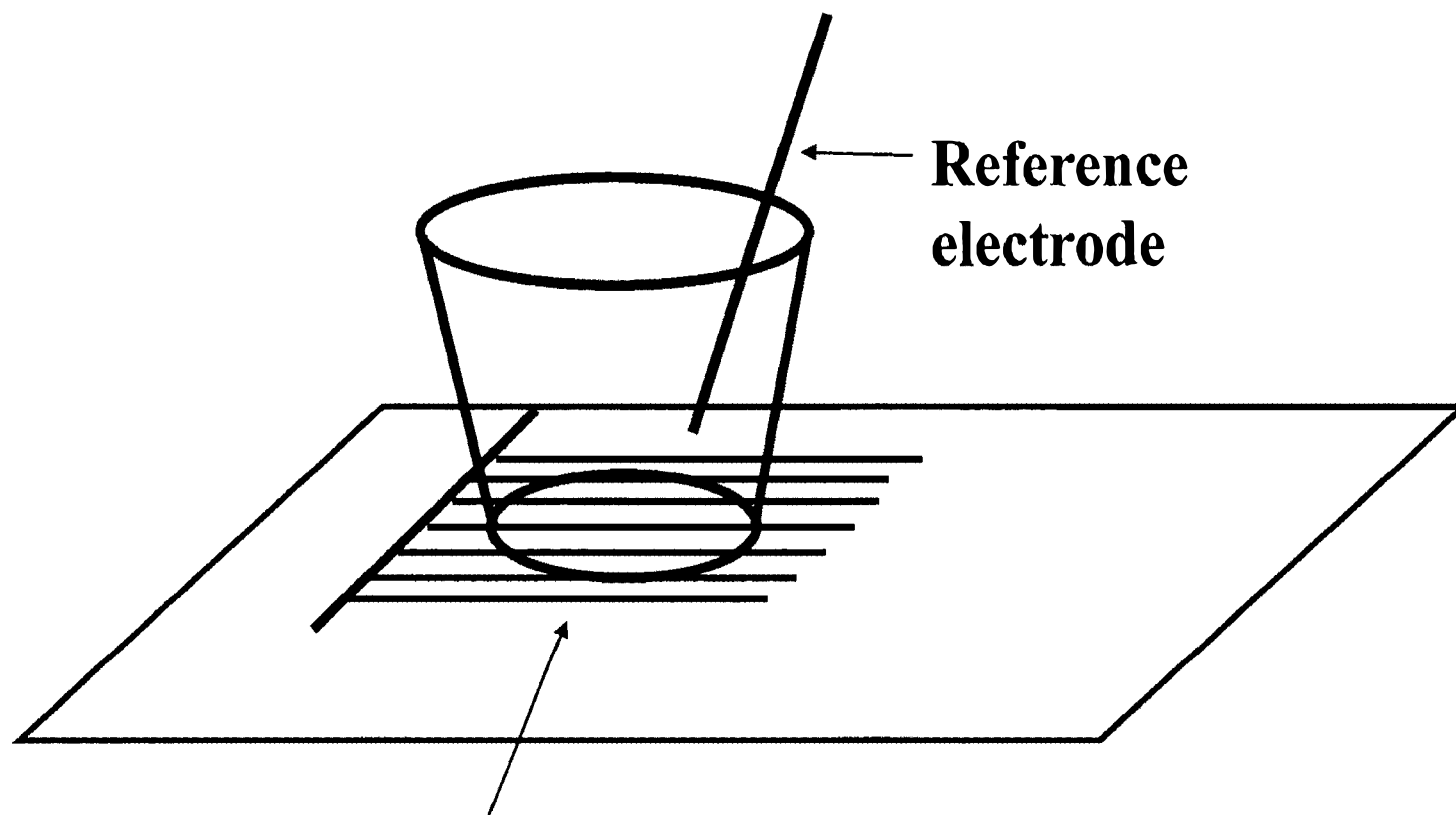


Figure 11. (continued)





Electrode structure on the substrate

Figure 12

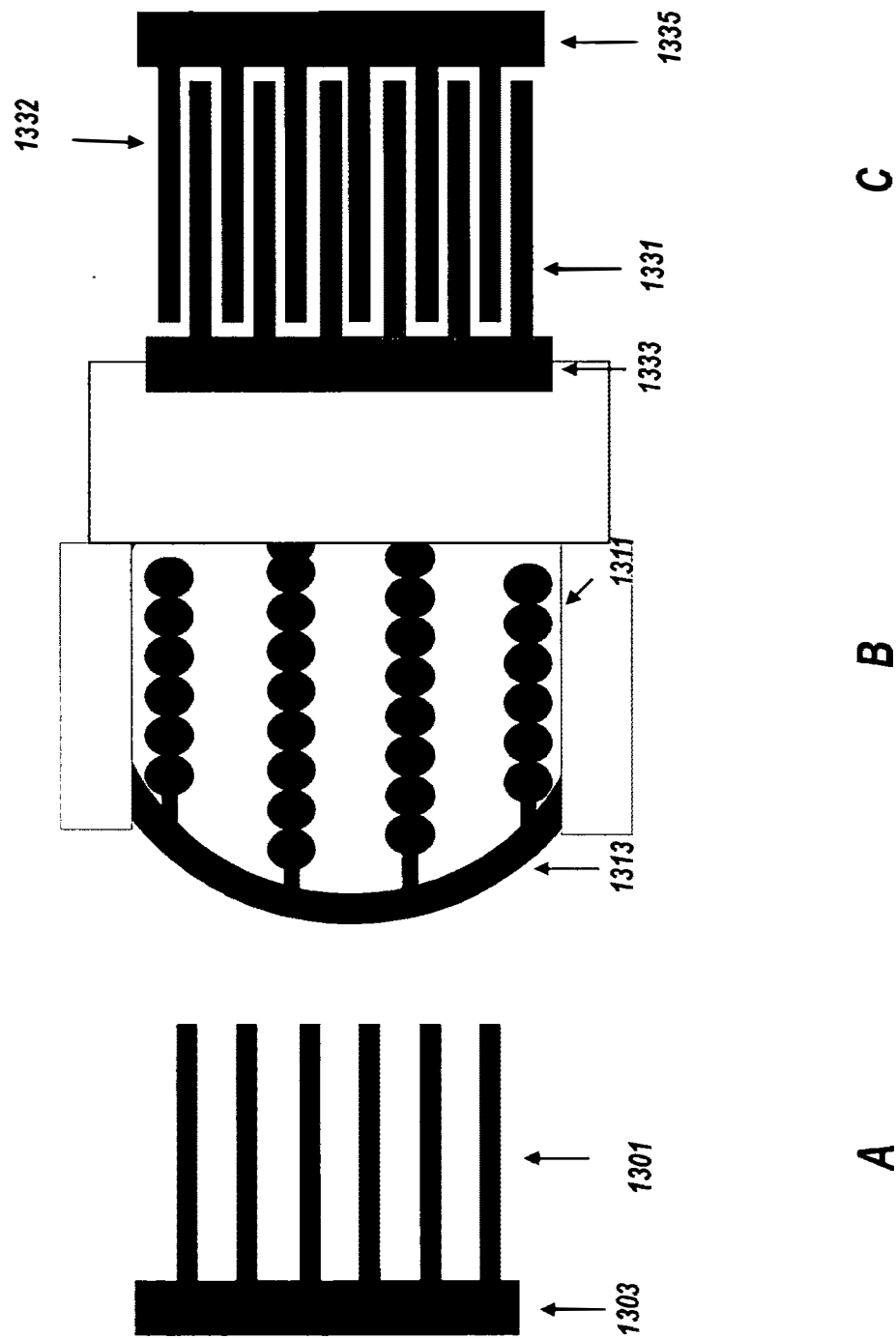
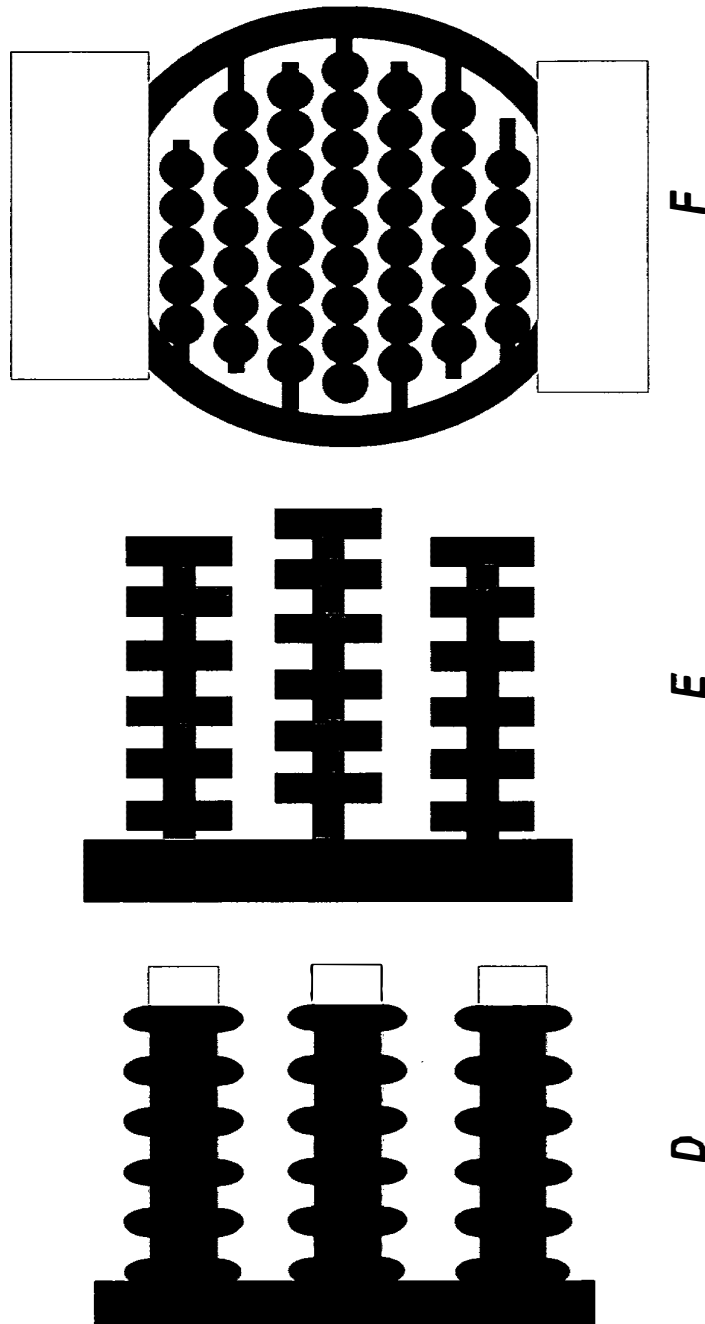


Figure 13

Figure 13 (continued)



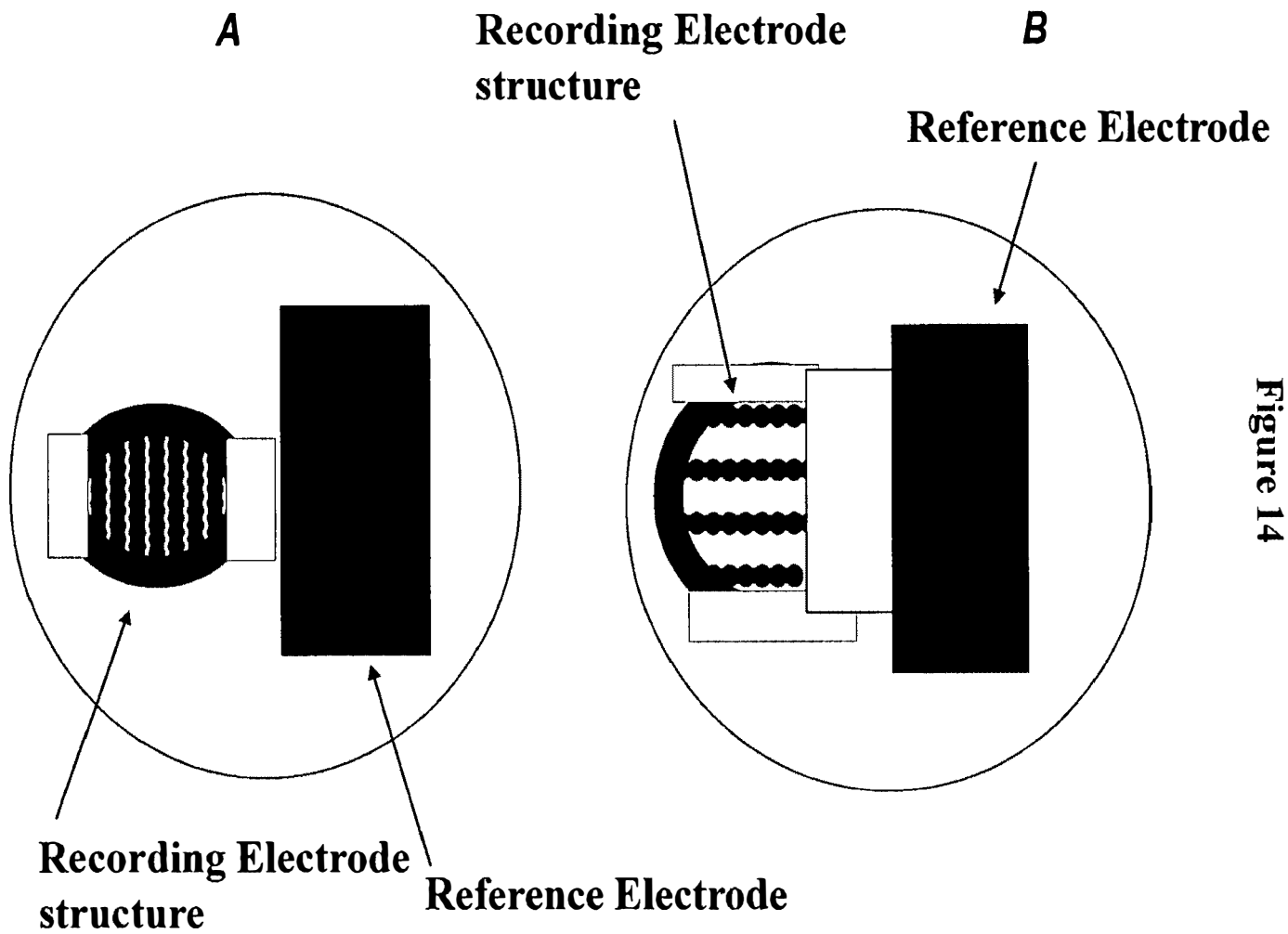


Figure 14

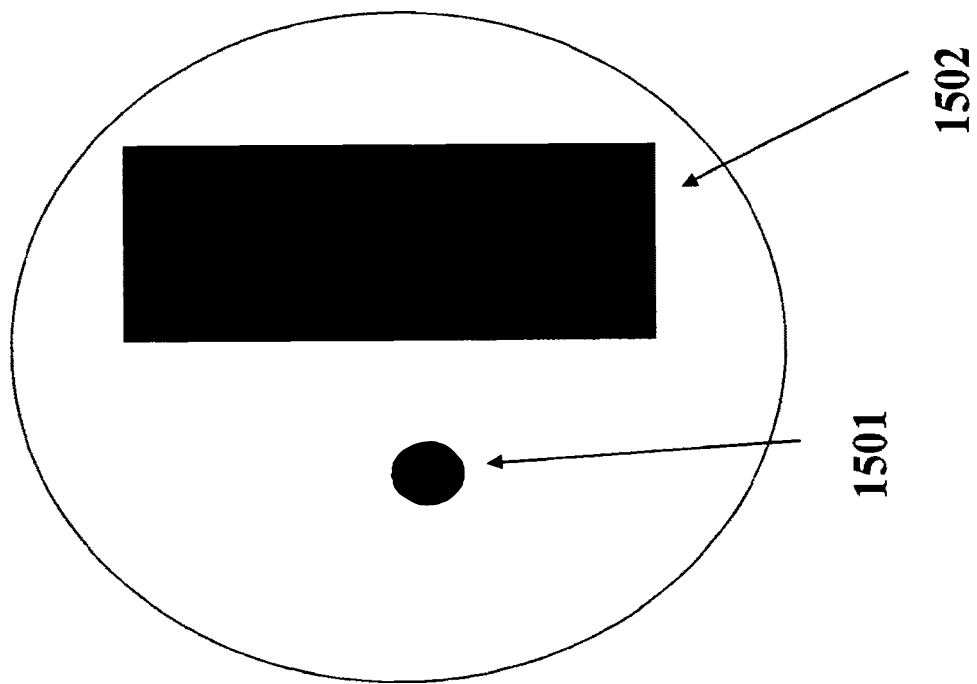


Figure 15

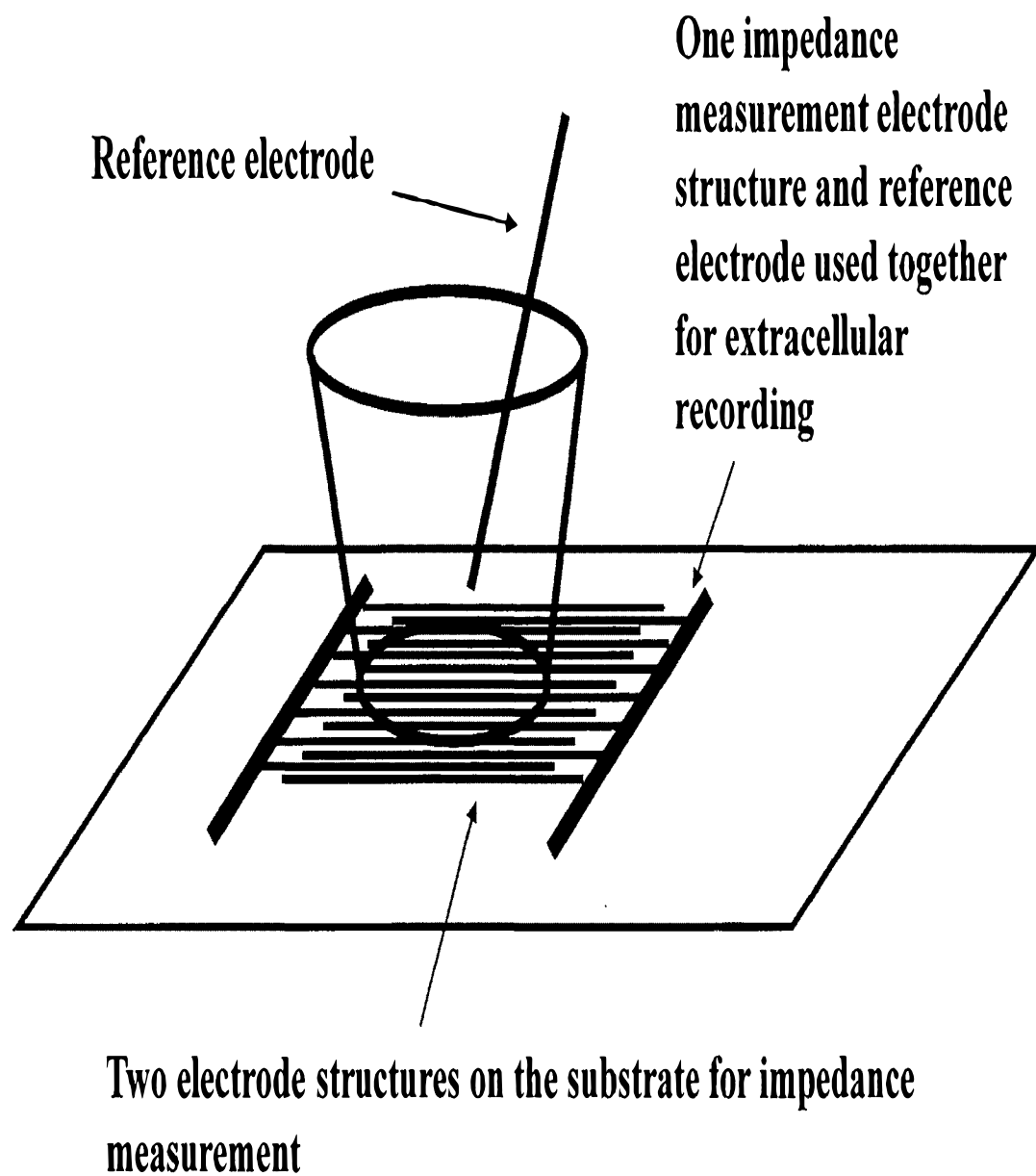


Figure 16

Figure 17

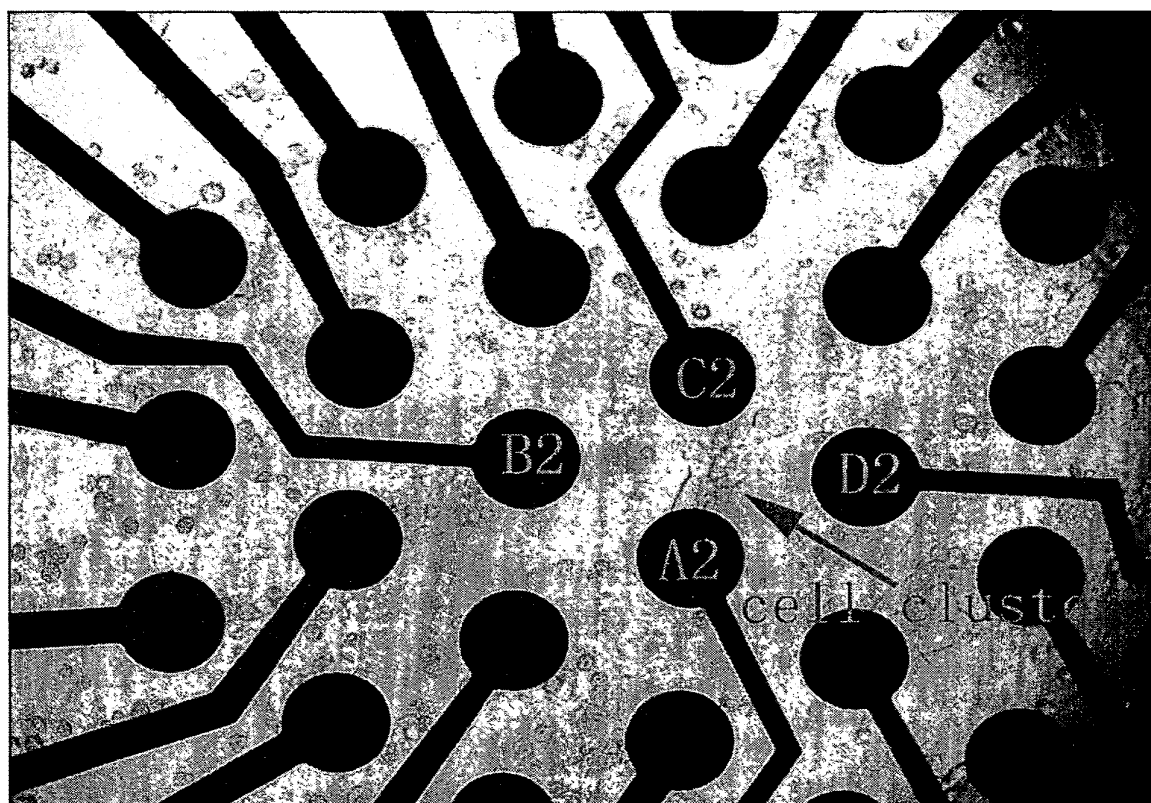


Figure 18

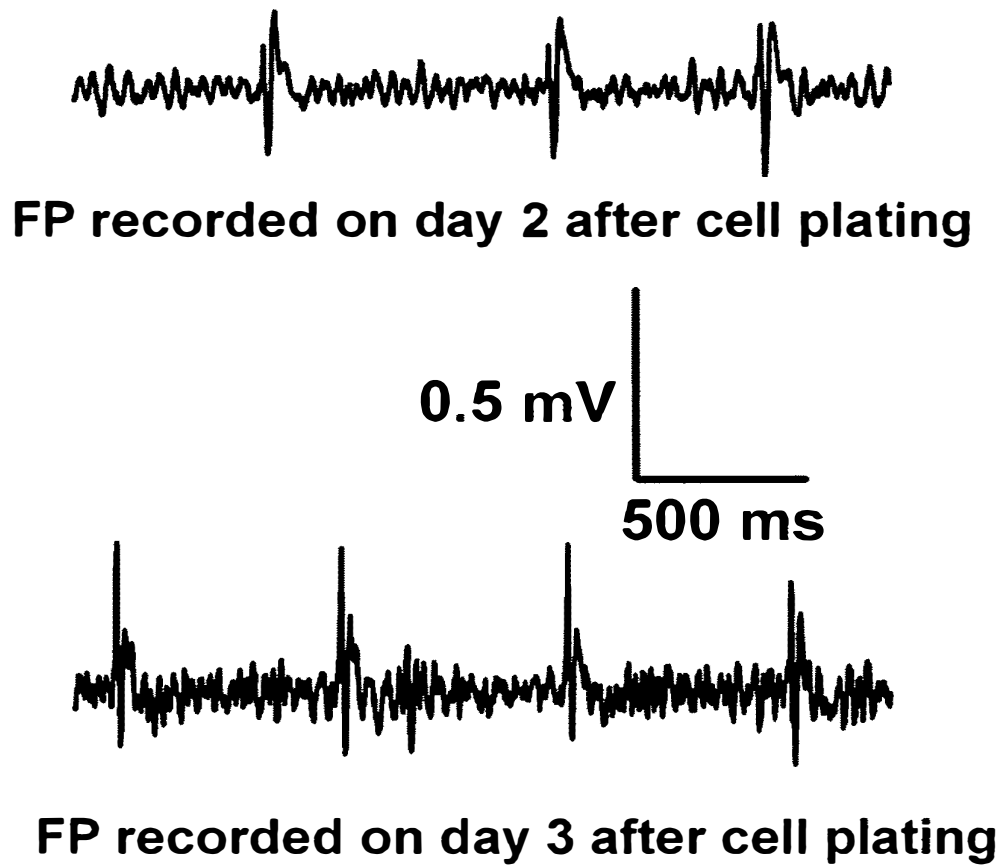


Figure 19

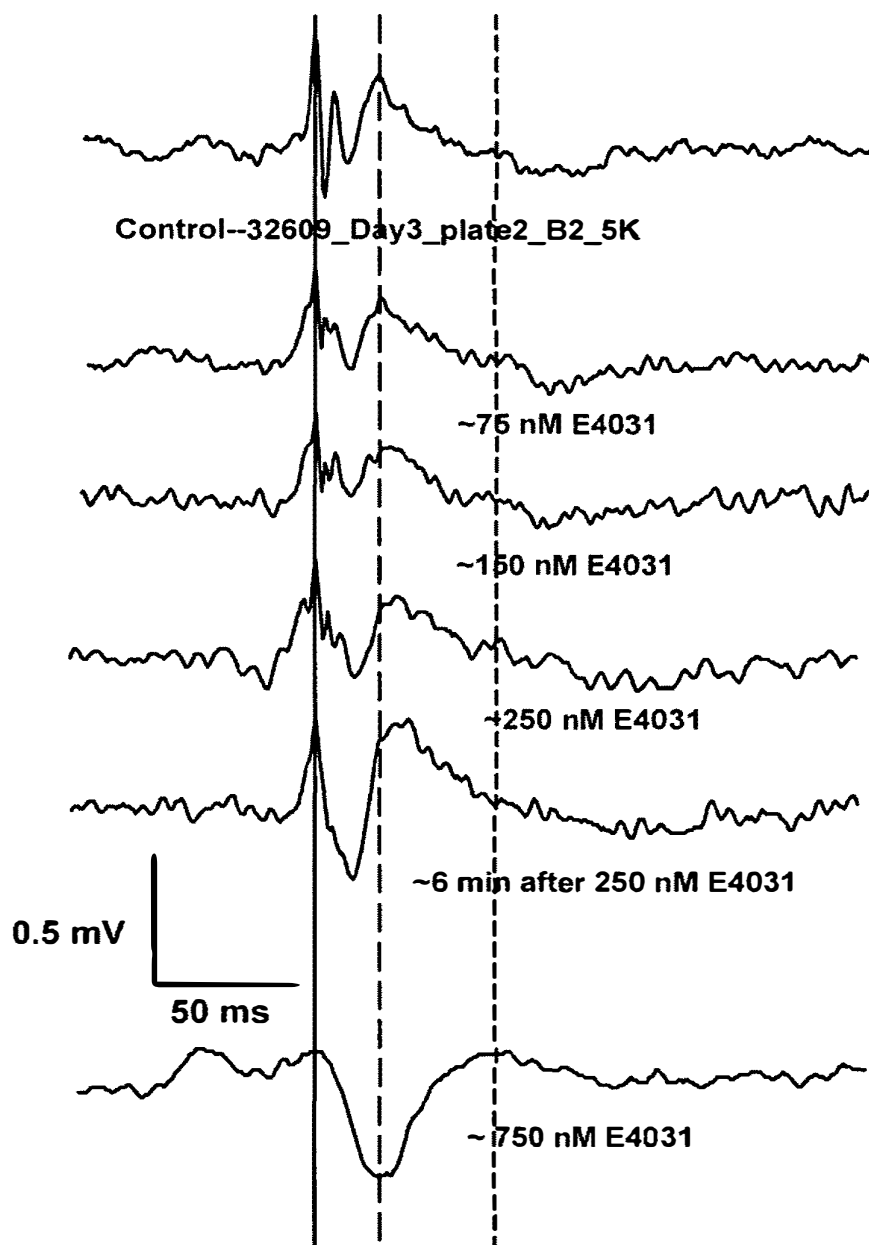


Figure 20

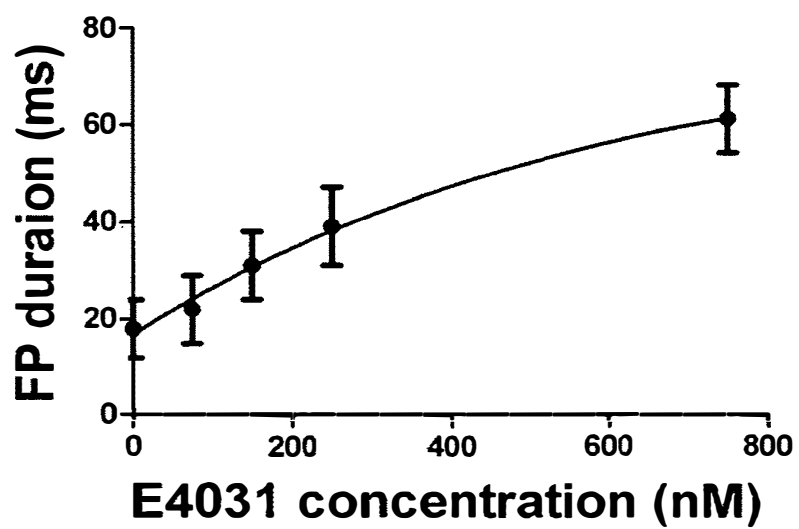


Figure 21

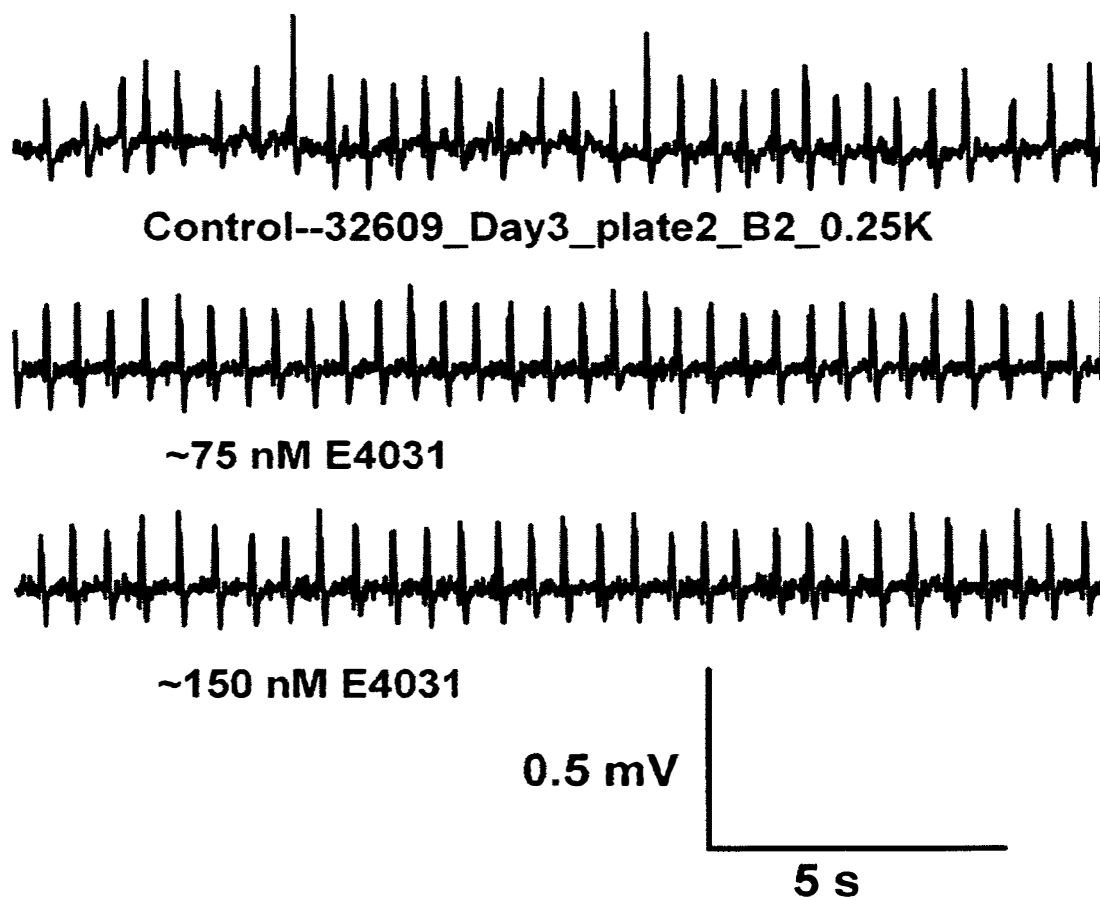


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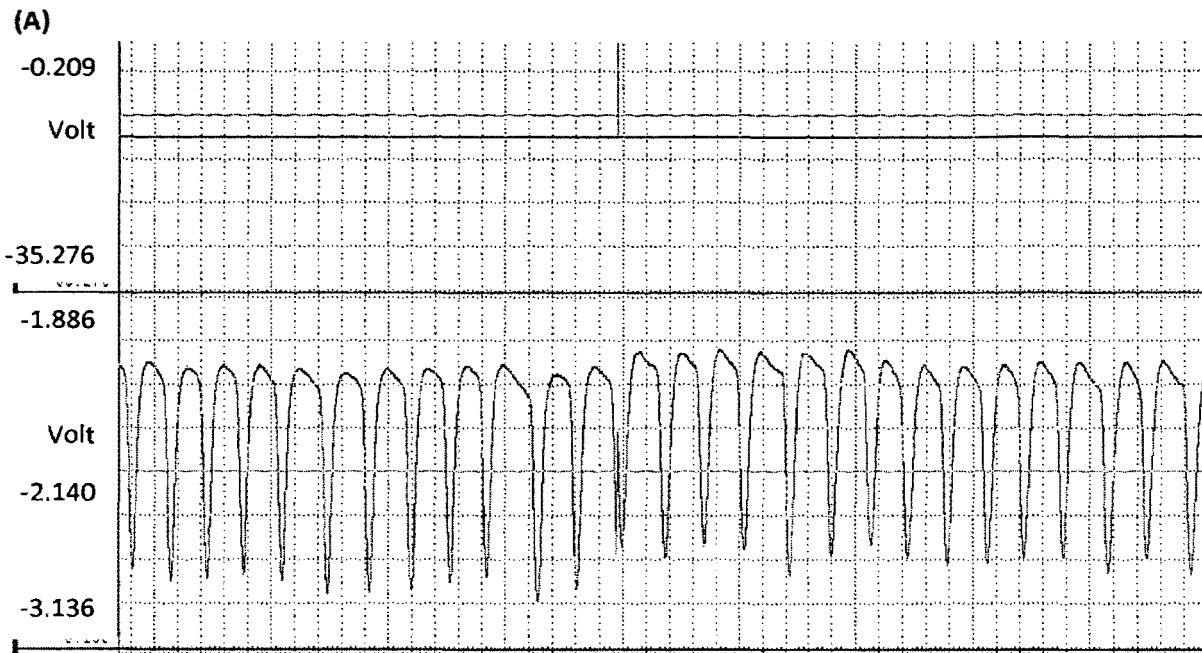


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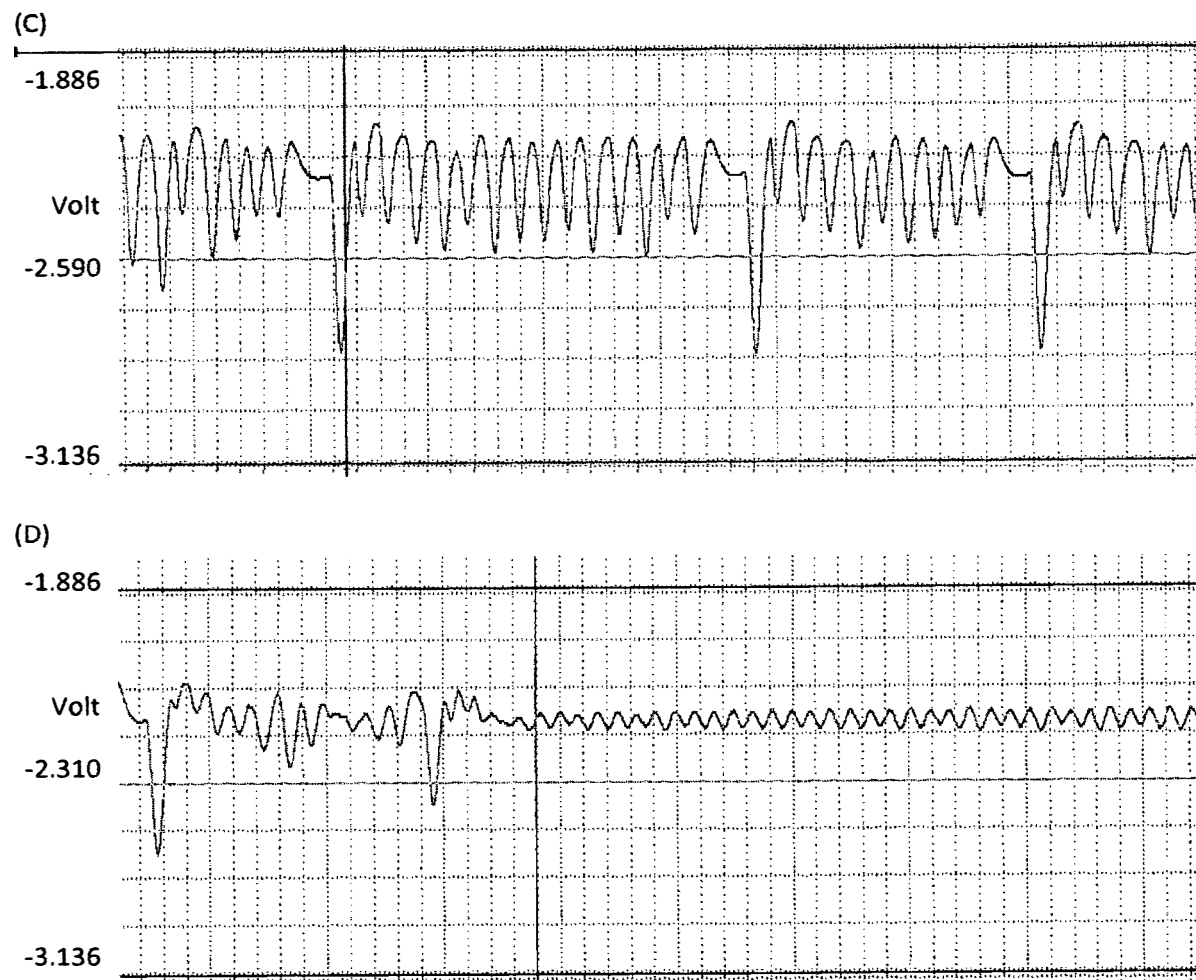


Figure 23.

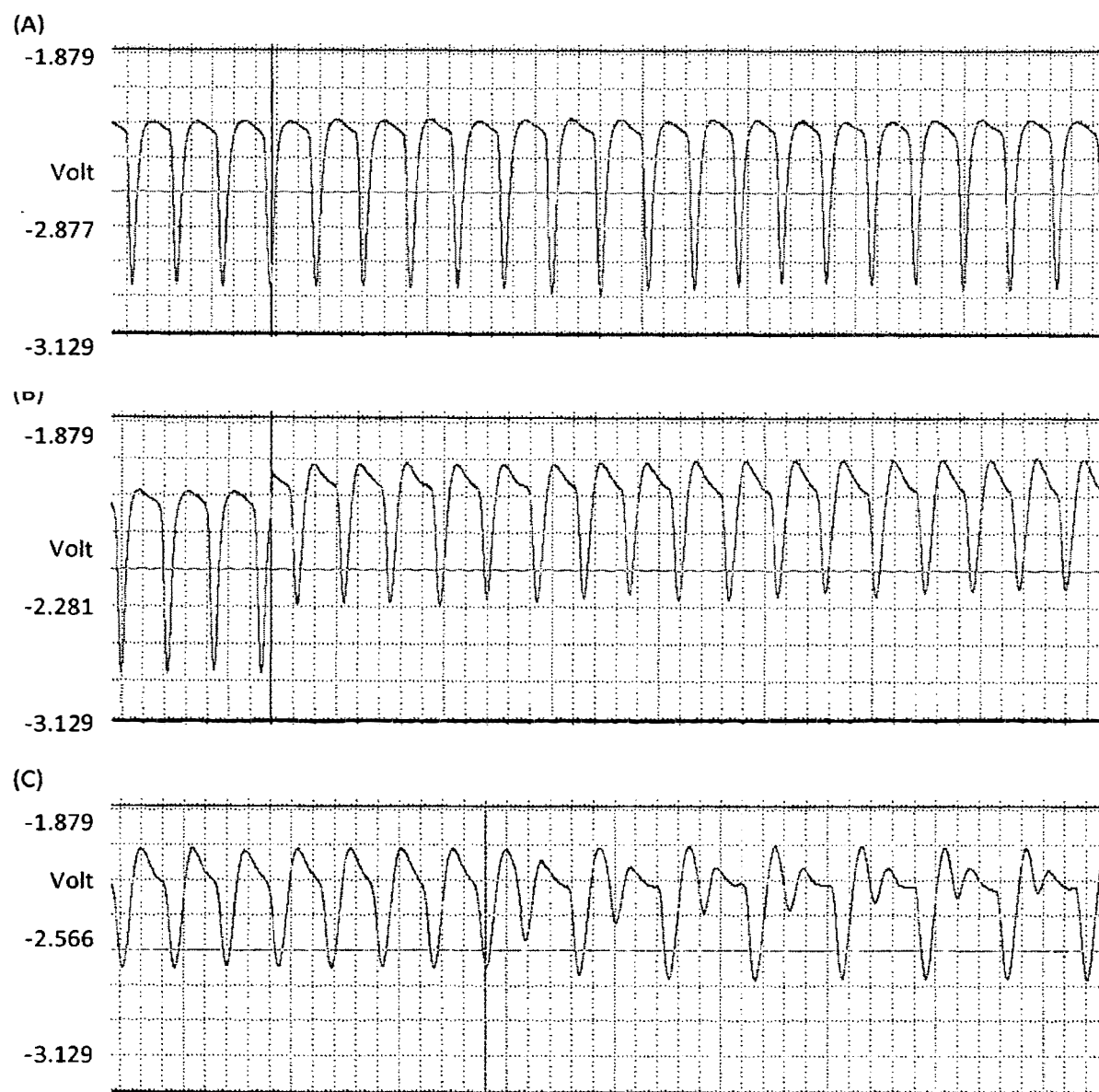


Figure 23.

(D)



(E)

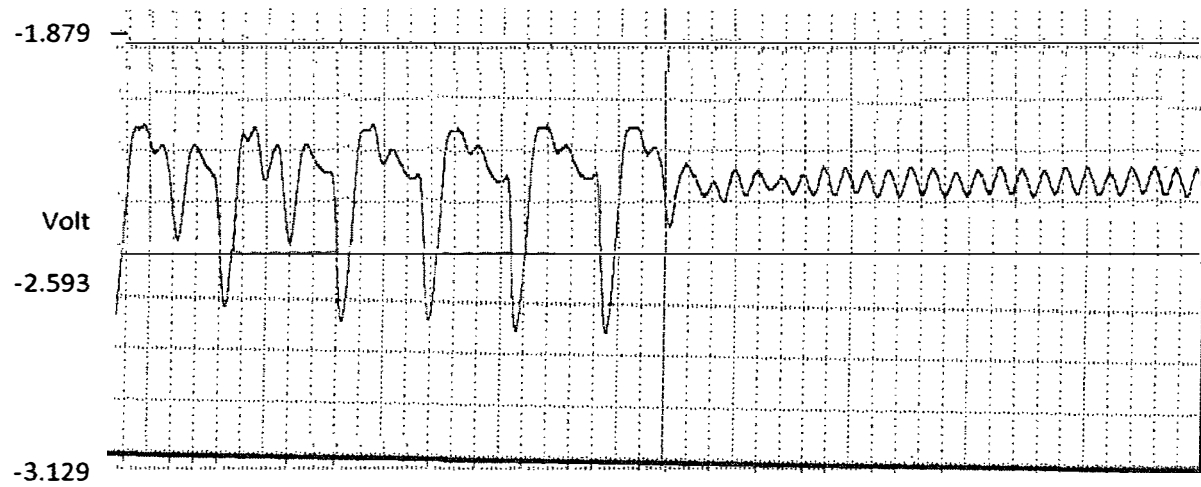
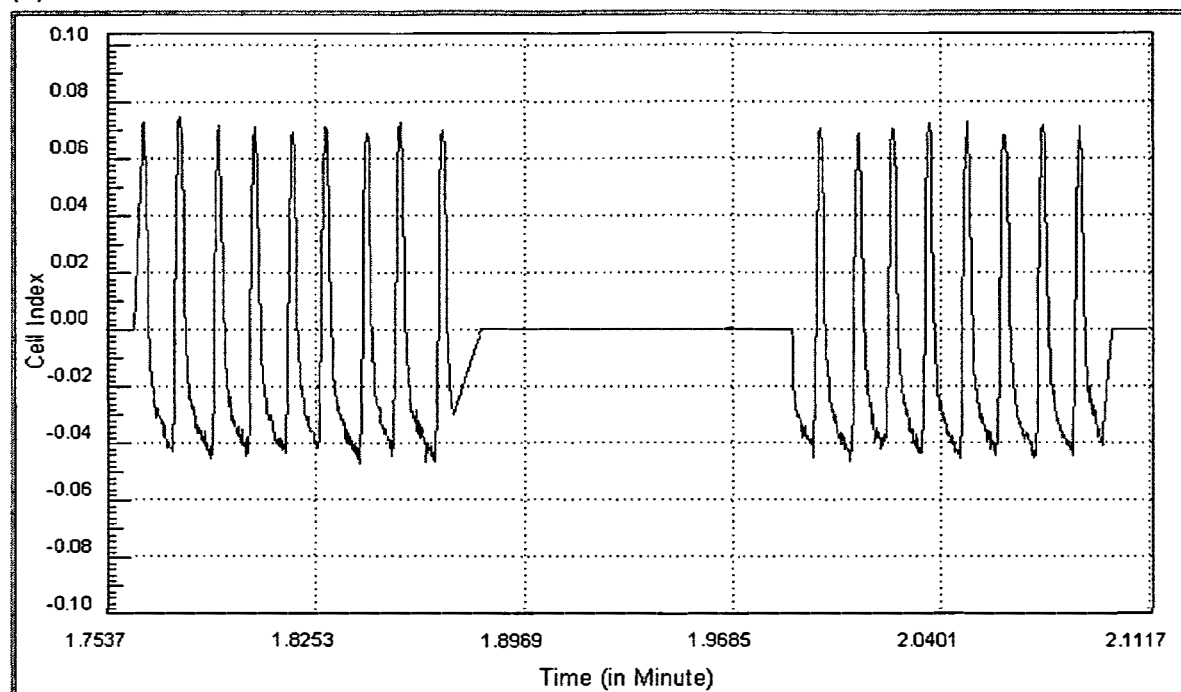


Figure 24.

(A)



(B)

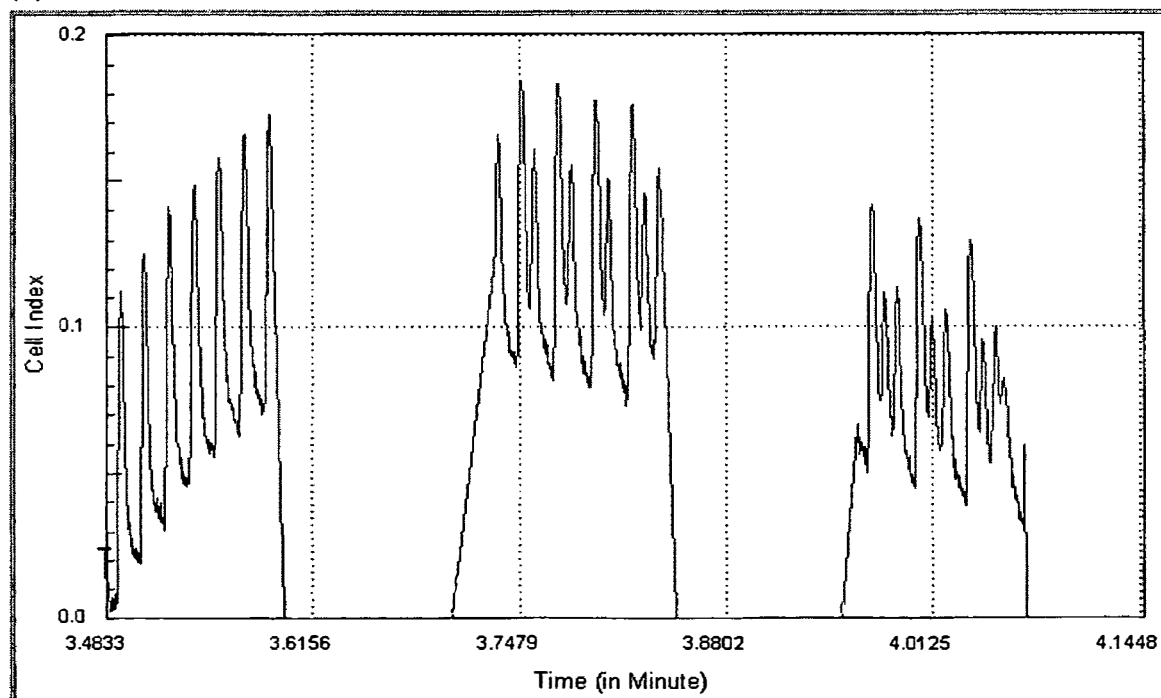
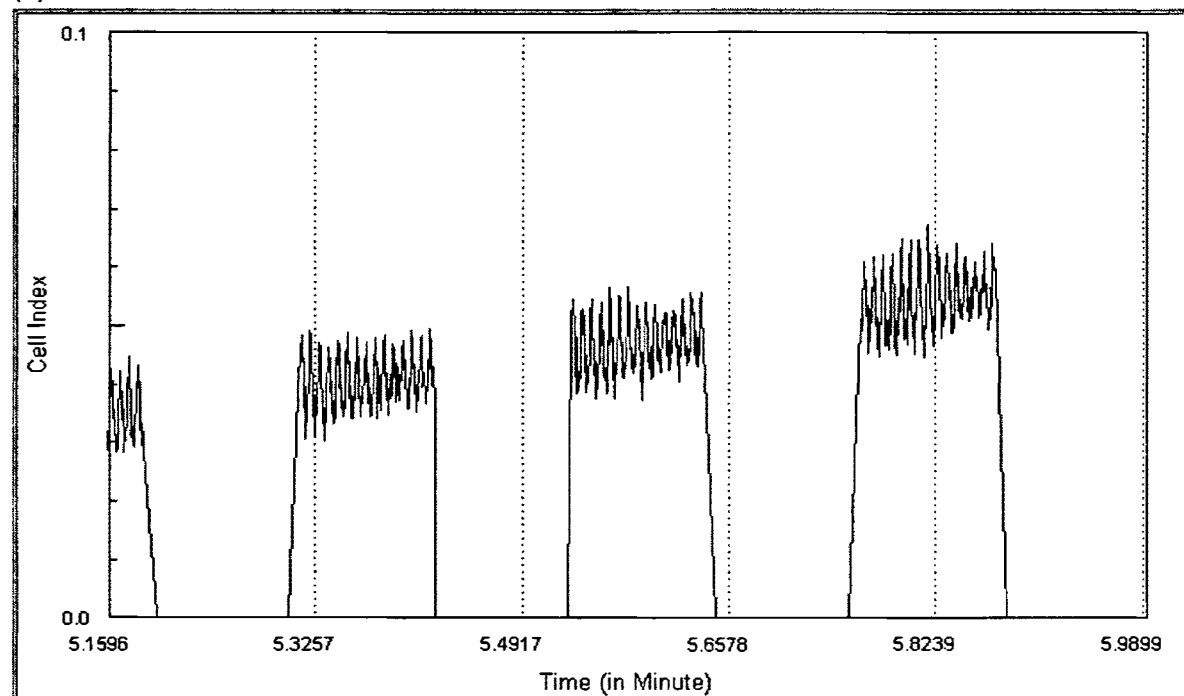


Figure 24.

(C)



(D)

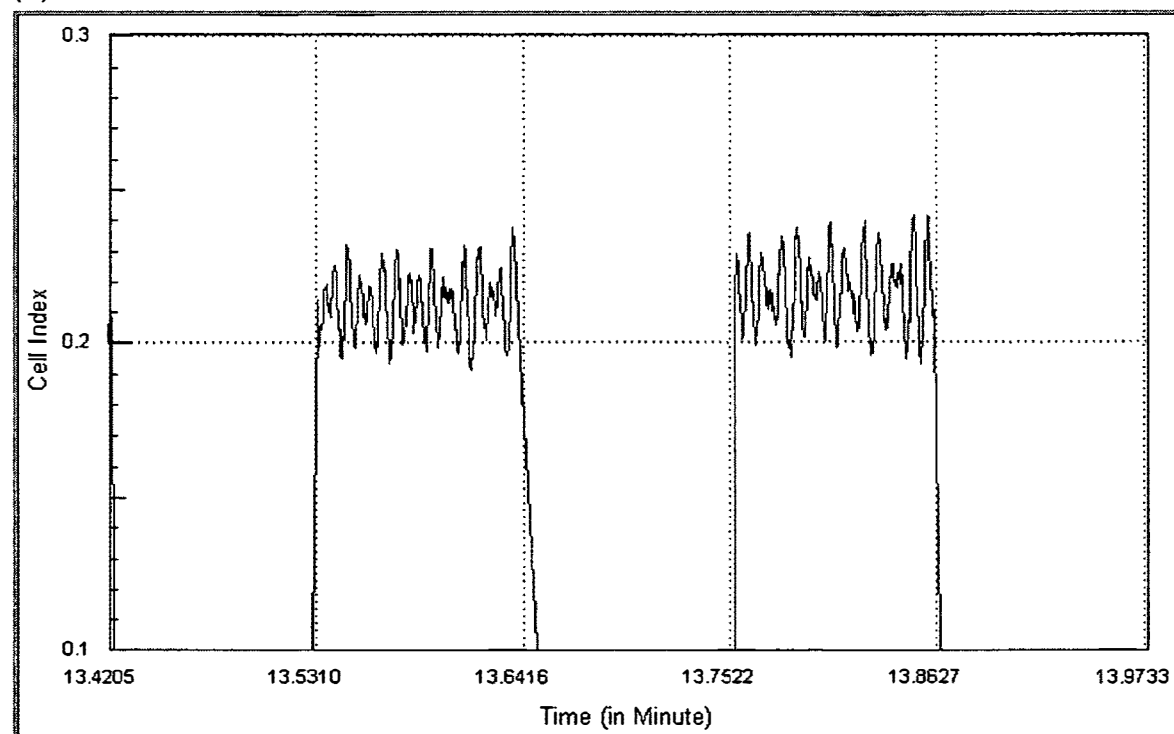
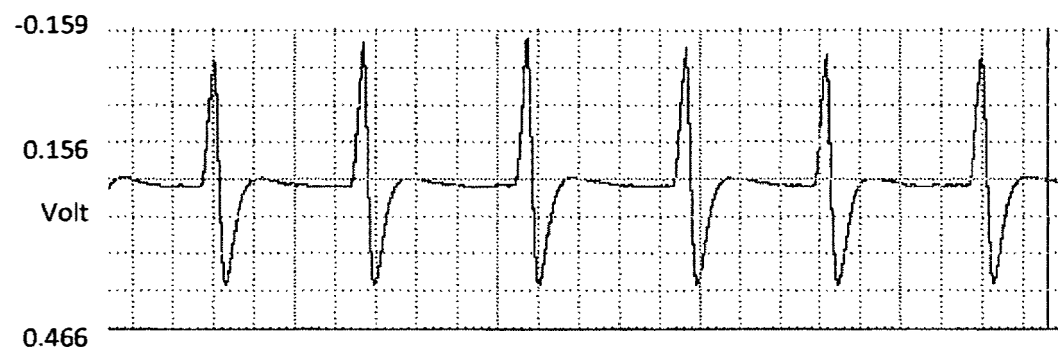


Figure 25.



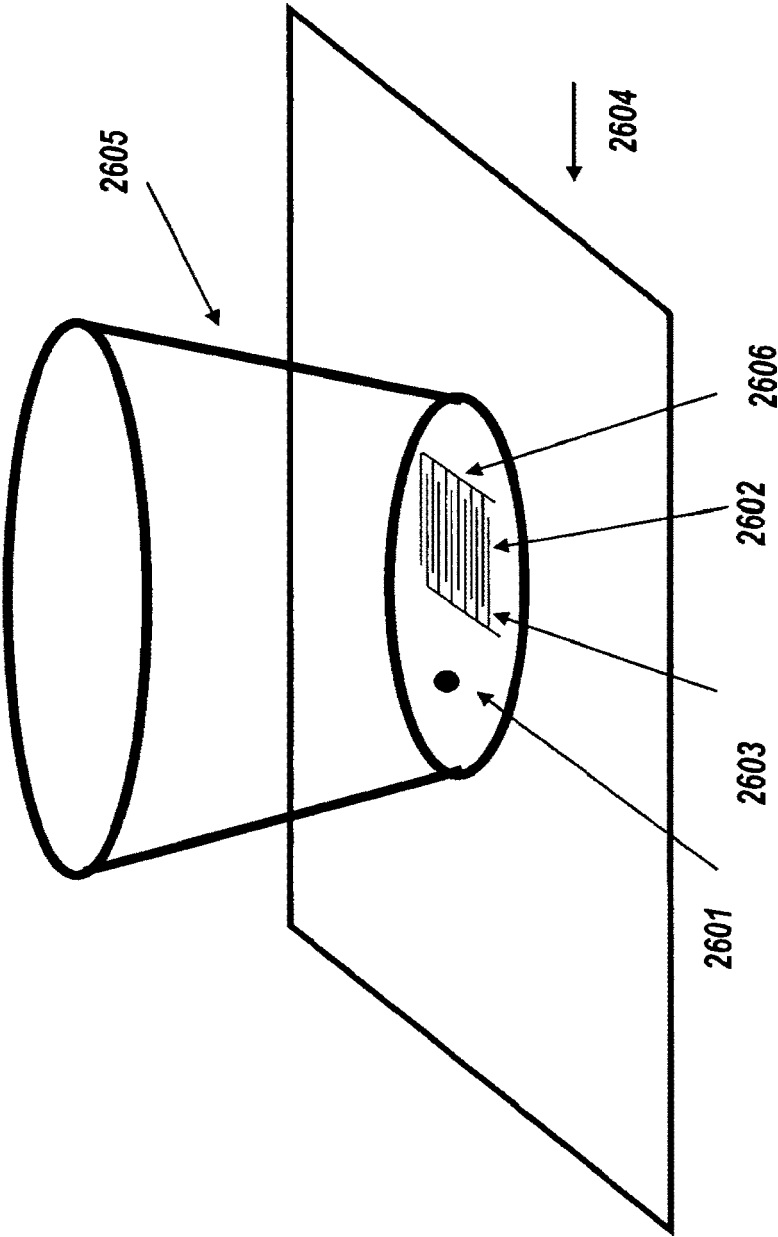


Figure 26

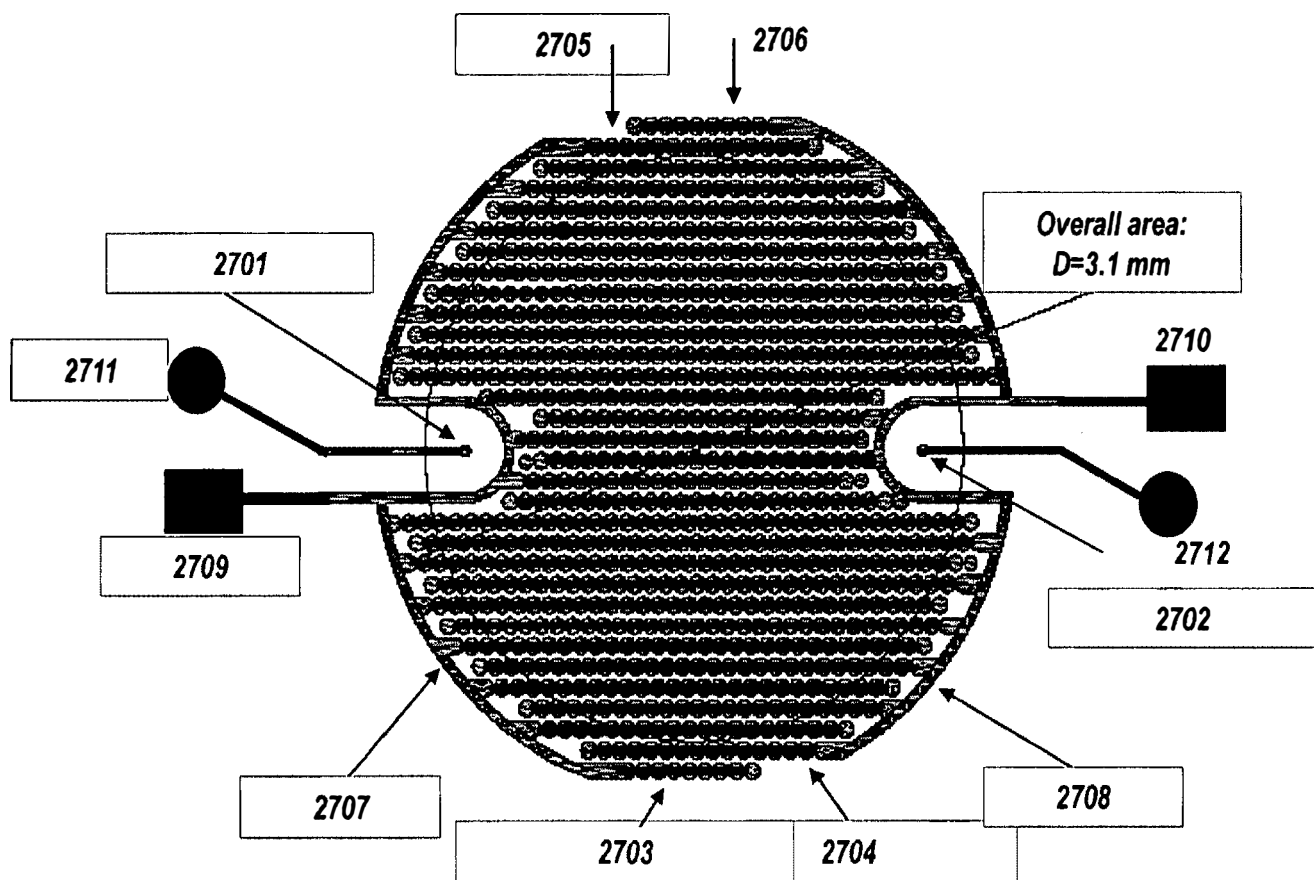


Figure 27

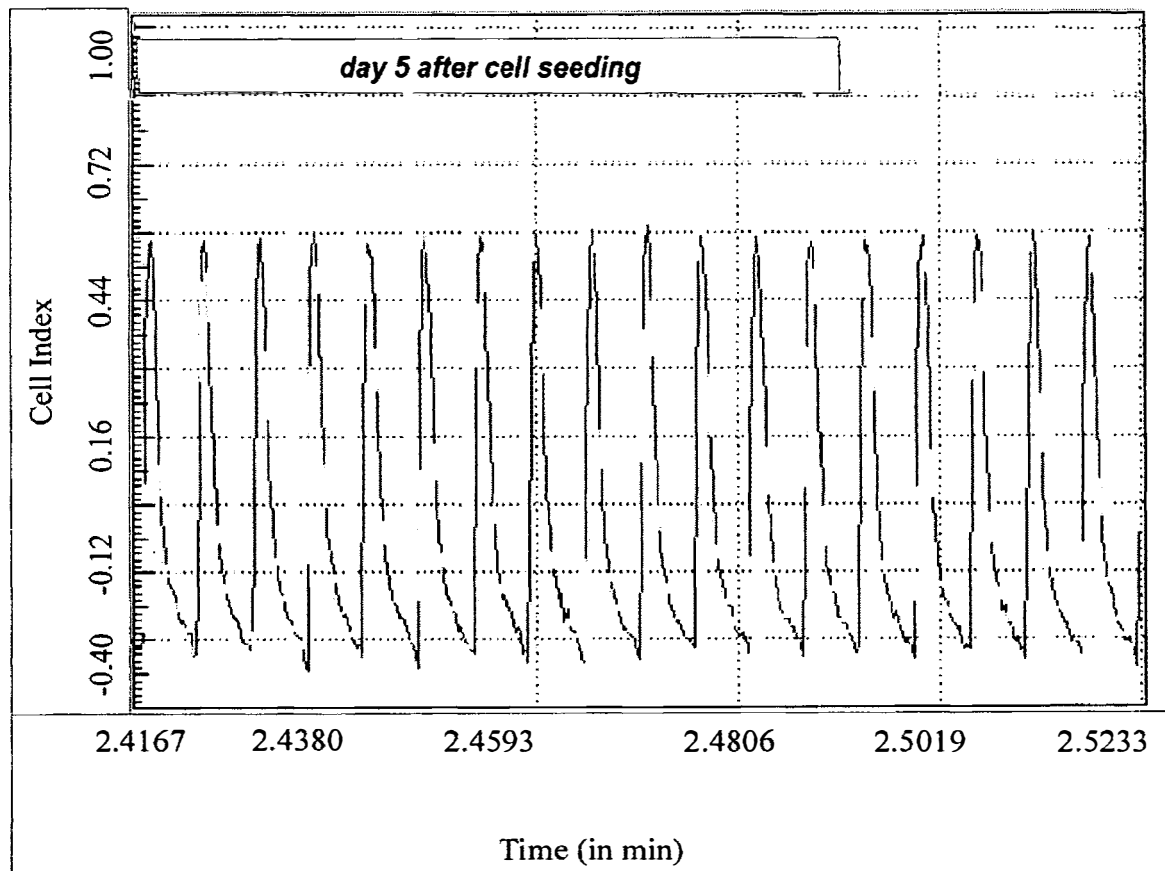
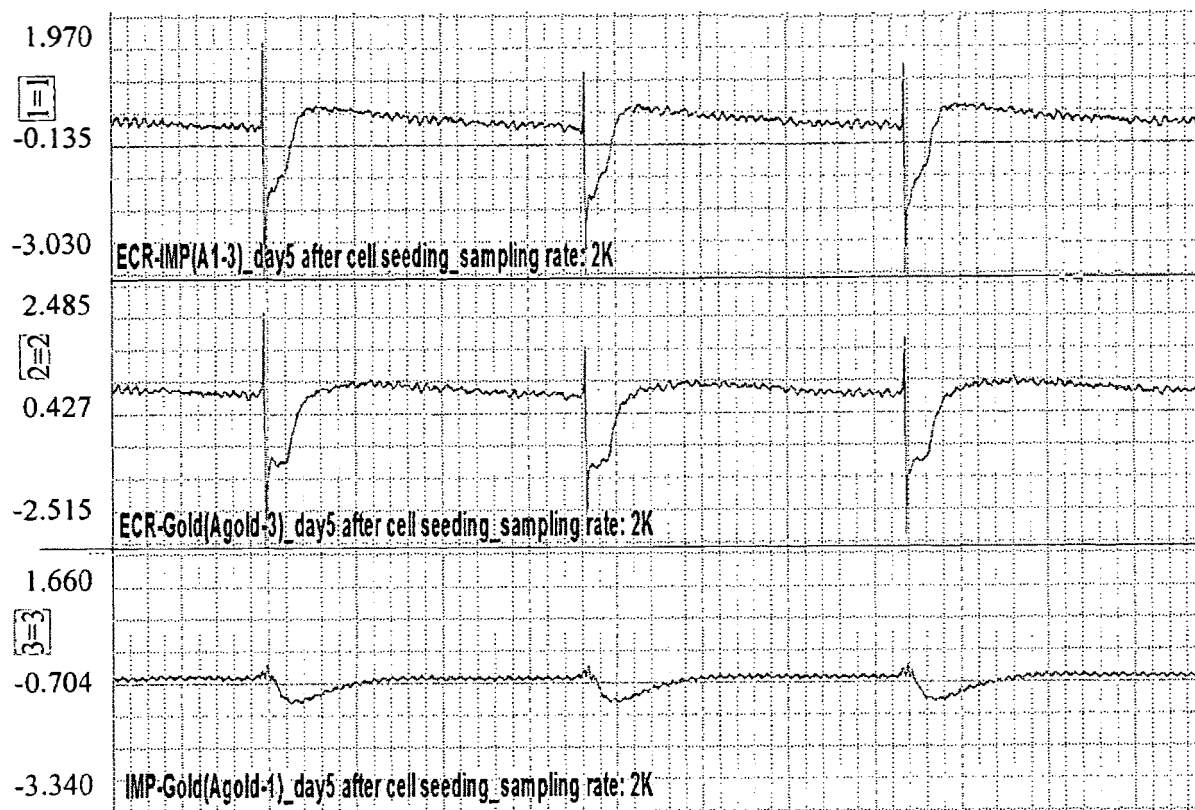
**Figure 28**

Figure 29.

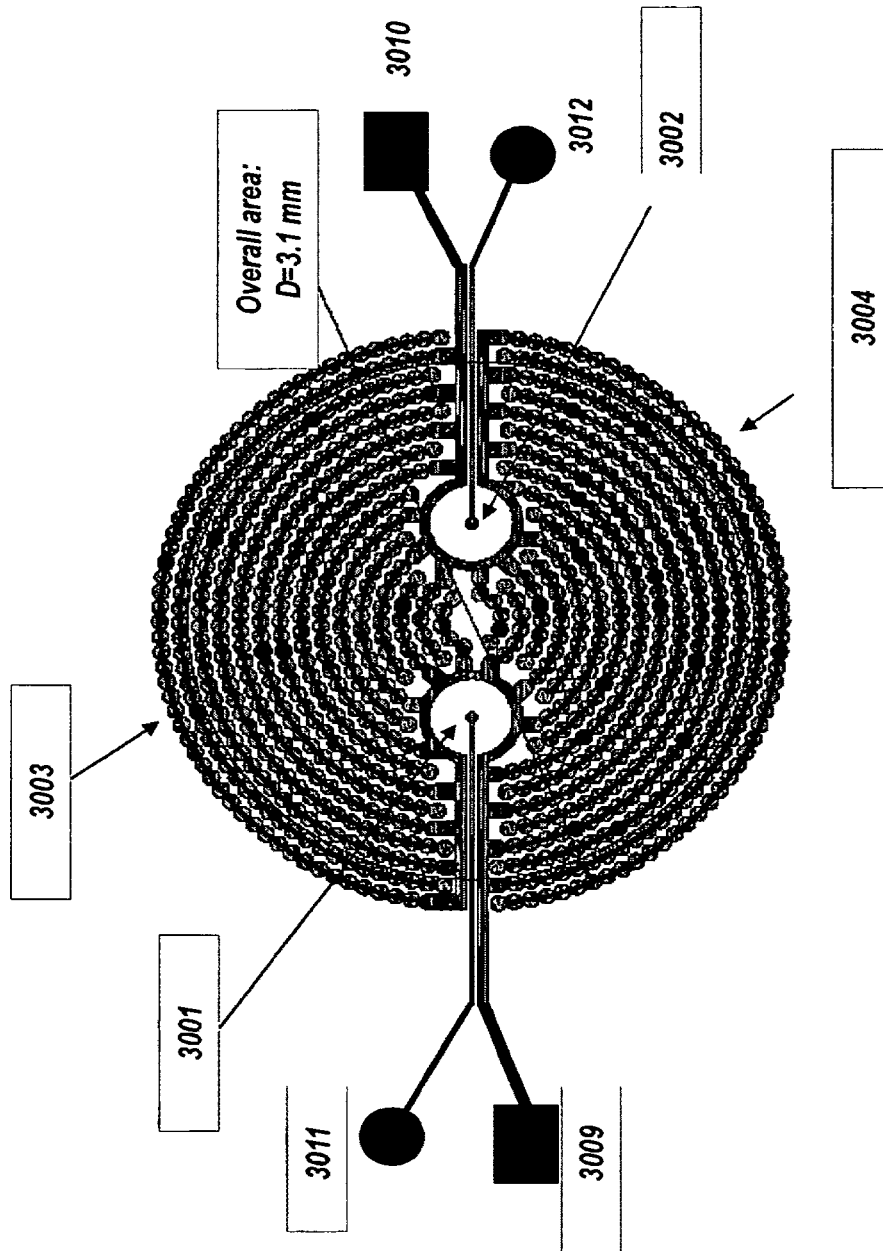


Figure 30

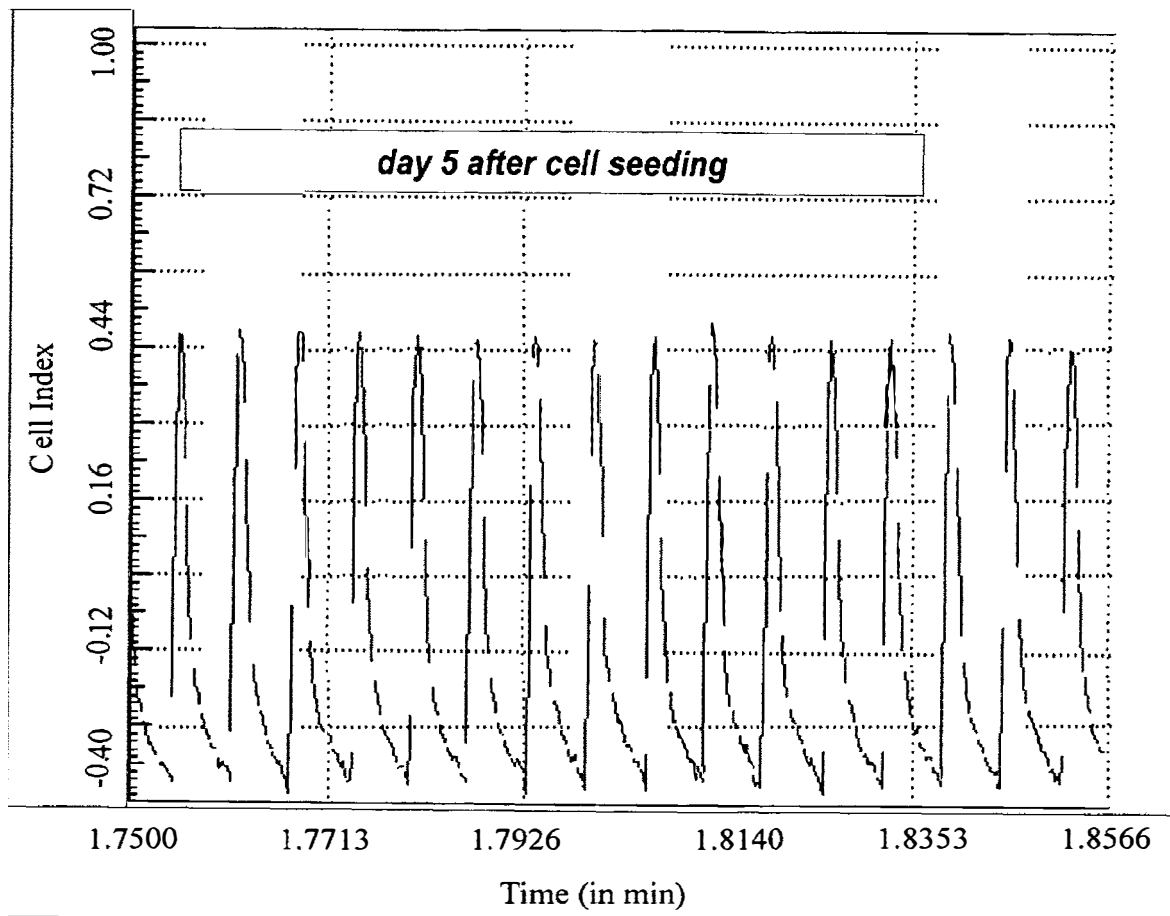
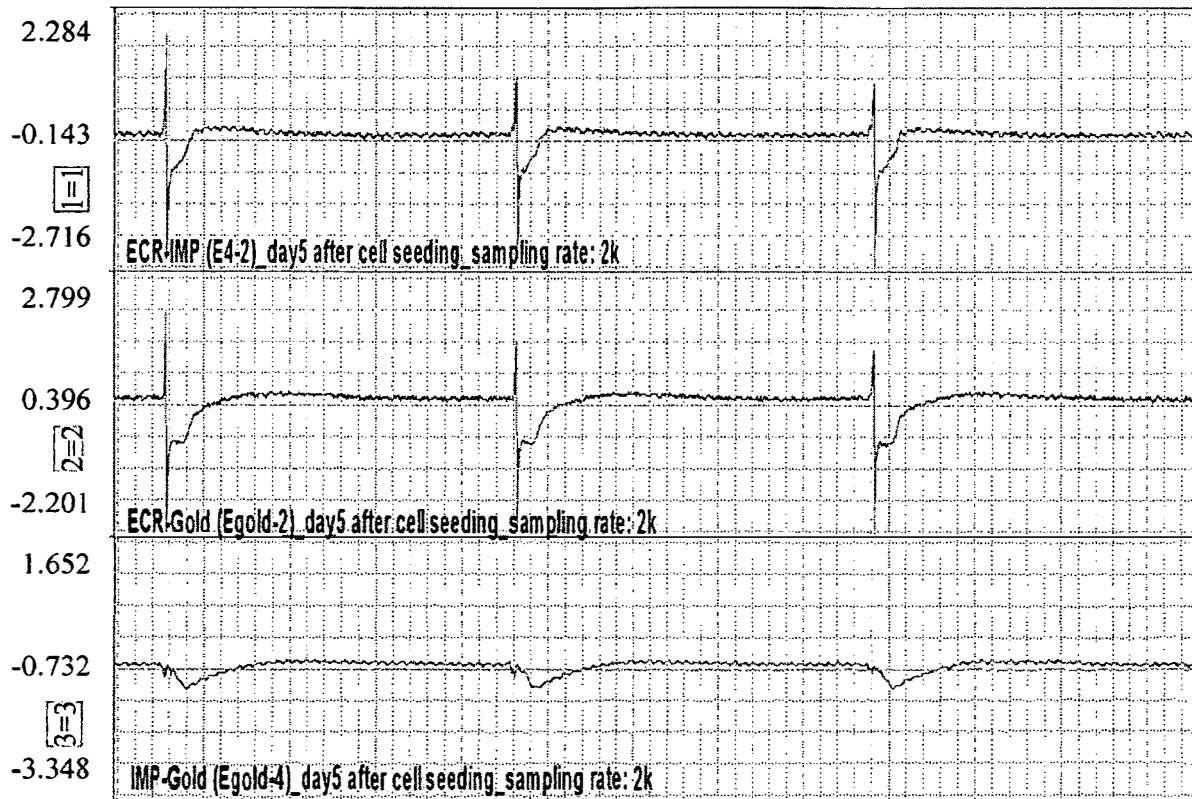
**Figure 31**

Figure 32.

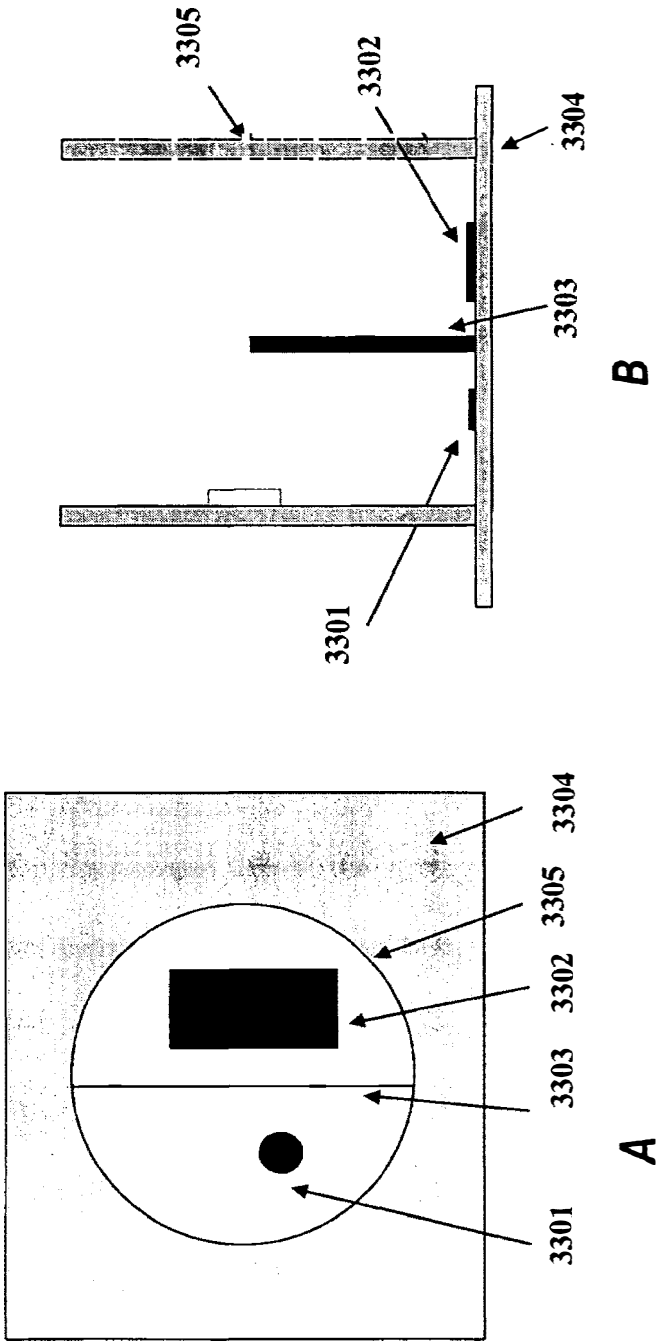


Figure 33

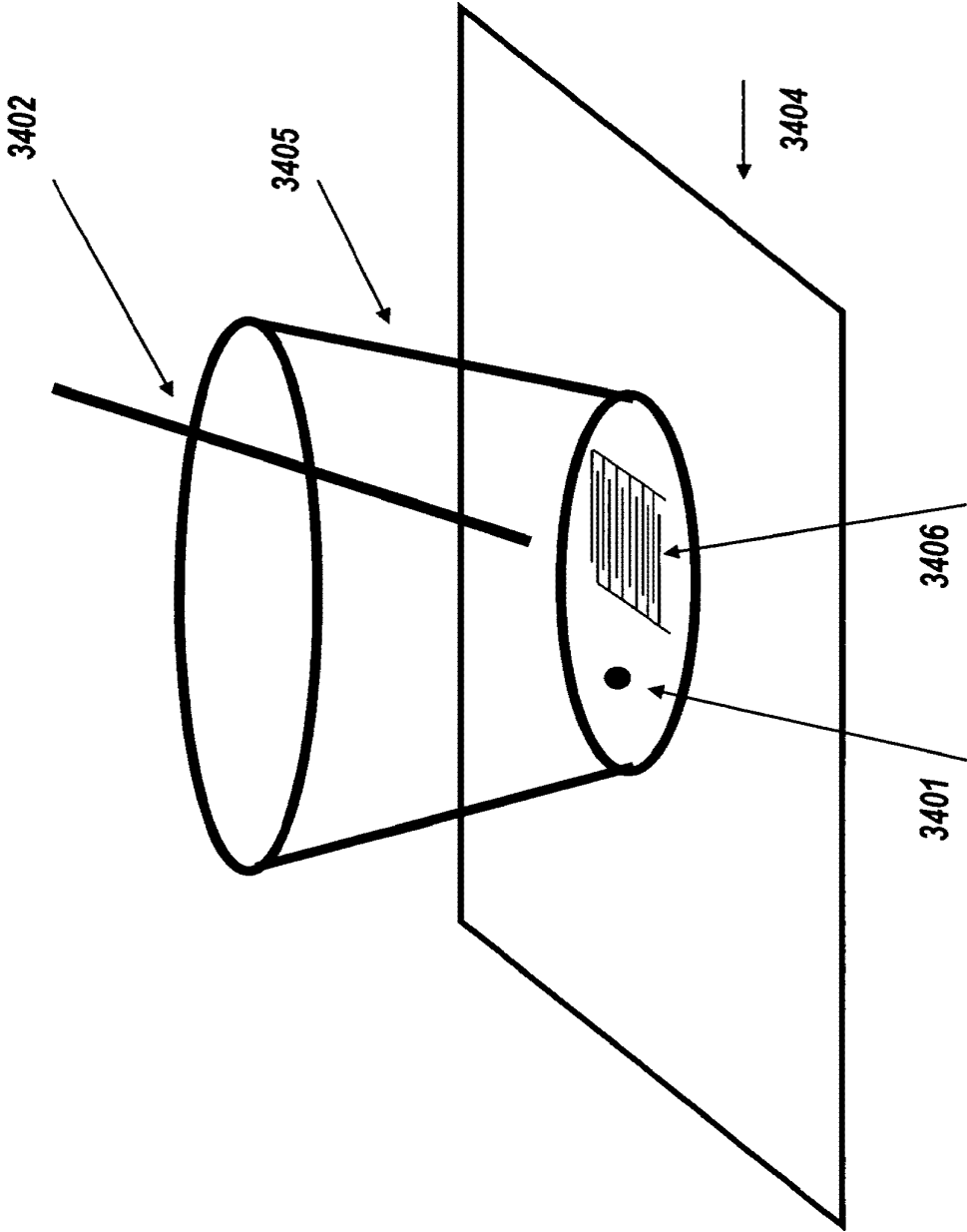


Figure 34

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2010/033801

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12M 1/34 (2010.01)

USPC - 435/287.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12M 1/00, 1/34, 13/00; G01N 27/00, 27/02 (2010.01)

USPC - 435/173.1, 173.4, 283.1, 287.1, 288.4; 436/149

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 040 345 B1 (SUGIHARA et al) 04 October 2000 (04.10.2000) entire document	1-4, 9-14, 19, 21-22, 24, 26, 28, 30, 32, 37
-		
Y		5-8, 15-18, 20, 23, 25, 27, 29, 31, 33-36, 38-41
Y	US 2008/0190783 A1 (HYLAND) 14 August 2008 (14.08.2008) entire document	5-8, 15-18, 23, 27
Y	US 7,459,303 B2 (WANG et al) 02 December 2008 (02.12.2008) entire document	20, 29, 31, 33, 36, 38, 41
Y	US 2006/0121446 A1 (ABASSI et al) 08 June 2006 (08.06.2006) entire document	25, 34, 35, 39, 40

☐ Further documents are listed in the continuation of Box C.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

17 June 2010

Date of mailing of the international search report

09 JUL 2010

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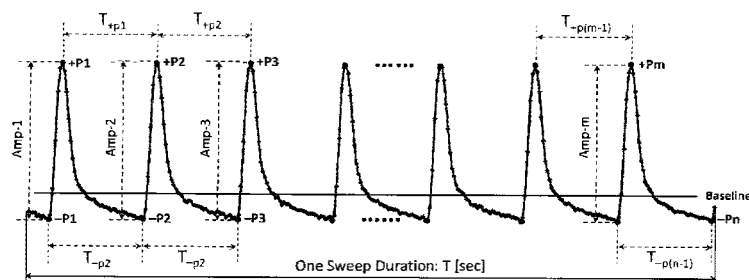


FIG. 7

(57) Abstract: A method of determining one or more beating parameters for use in cardiomyocyte beating analysis including: providing a cell analysis device including wells, each well including a sensor capable of monitoring beating of cardiomyocytes in millisecond time resolution; adding cardiomyocytes to the wells; monitoring the beating of the cardiomyocytes in millisecond time resolution to obtain a plurality of beating measurements; and calculating one or more beating parameters from the plurality of beating measurements.

**DATA ANALYSIS OF IMPEDANCE-BASED CARDIOMYOCYTE-BEATING
SIGNALS AS DETECTED ON REAL-TIME CELL ANALYSIS (RTCA)
CARDIO INSTRUMENTS**

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority to US patent application serial no. 61/345,867 filed on May 18, 2010.

10 TECHNICAL FIELD

The invention relates to cell based assays and their use for monitoring cardiomyocyte beating and more specifically to methods for determining cardiomyocyte beating parameters and their use to identify compounds having
15 potential cardiotoxic effects.

BACKGROUND OF THE INVENTION

Cardiac safety pharmacology is the study of the potential undesirable
20 pharmacodynamic effects of a substance on heart function in relation to exposure to the substance in the therapeutic range and above. Cardiac safety is a major concern in current drug development. Since 1981, at least 10 blockbuster drugs have been withdrawn from the market due to cardiac liability, defined as potentially undesirable effects on heart function. Furthermore, cardiac safety is a major reason for late stage
25 attrition of drug candidates during development.

There are three non-mutually exclusive ways that non-cardiac drugs may lead to cardiac liability. Directly, cardiotoxic drugs are drugs which cause damage via necrosis or apoptosis, such as anthracyclines. Pro-arrhythmic drugs are drugs which induce arrhythmia. Indirectly, cardiotoxic drugs are drugs which indirectly affect
30 cardiac function, such as by causing narrowing of the arteries.

Directly, cardiotoxic drugs directly affect the viability of cardiomyocytes and therefore heart function. A prominent class of drugs in this category is chemotherapeutic drugs, such as anthracyclines. Mortality due to cardiac disease is thought to be 8-fold higher for survivors of childhood cancers who have received

chemotherapy. These drugs are thought to disrupt iron metabolism, generating harmful oxygen radical species which ultimately cause mitochondrial damage and apoptosis.

Pro-arrhythmic drugs induce arrhythmia. Normal synchronized contractile activity of cardiomyocytes is the result of orchestrated ion currents passing across the cell membrane via ion-specific channels and coupling with the specialized cytoskeleton. Disturbances in the ionic movement of interference with ion channel activities may lead to arrhythmia. It is believed that one of the primary targets of pro-arrhythmic drugs is the ERG channel, which is responsible for delayed repolarization of cardiomyocytes. ERG channel blockage may lead to QT elongation and this may cause a fatal form of ventricular arrhythmia called Torsades de Pointes (TdP). Between 1990 and 2006, 10 blockbuster drugs have been withdrawn from the market due to induction of TdP. The drugs that have been associated with cardiac arrhythmia and removed from the market are prenylamine, terodiline, sparfloxacin, sertindole, terfenadine, astemizole, grepafloxacin, cisapride, droperidol, and levacetylmethadol.

Excitation-contraction coupling (ECC) is a term used to describe the physiological process of converting an electrical stimulus to a mechanical response. The process is fundamental to muscle physiology, wherein the electrical stimulus may be an action potential and the mechanical response is in the form of contraction. Although ECC has been known generally over half a century, it is still an active area of biomedical research.

Cardiomyocytes are specialized muscle cells of the myocardium that are capable of excitation-contraction coupling. Cardiomyocytes are commonly used in biomedical research to assess the cardiotoxicity of potential drugs or treatments. Two conventional approaches to assess cardiotoxicity are primarily used. A first approach involves isolation of cardiomyocytes directly from a mammalian species such as rats and dogs followed by electrophysiological studies on the isolated cardiomyocytes. However, this approach suffers from being extremely labor-intensive, time consuming and costly and at the same time not very amenable to the high throughput demands of pharmaceutical industry. An alternative approach utilizes cell-based assay models, which heterologously express specific ion channels such as hERG channels or voltage-gated calcium channels. These cardiac ion channels have been envisioned as possible molecular targets through which drugs could induce cytotoxicity. These cell-based systems allow assessment of drug-channel interaction by monitoring the effect

of the drug on currents produced by different channels in cultured cells using a technique known as “patch clamping.” Patch clamping isolates regions of the cell membrane containing channel proteins and measures changes in electrical potential difference. However, use of this method in high throughput requires automation of
5 patch clamping in an array format with reliable giga seal, which even though is becoming increasingly available, is not yet widespread. In addition, cardiac toxicity may occur by other mechanisms that could be possibly missed by this type of targeted approach.

An alternative to *in vitro* ion-channel recording assays as well as the labor-
10 intensive isolation of primary tissue is the differentiation of embryonic stem (ES) cells into cardiomyocytes. The utility of ES cells as a treatment for various chronic diseases has received much attention in recent years. Mammalian ES cells are self-renewing cells derived from the inner cell mass of a blastocyst stage embryo which can be differentiated into multiple different cell types. It has been demonstrated that
15 the mouse ES cells as well as human ES cells can be differentiated into cardiomyocytes which retain the ability to beat in culture. Differentiation of ES cells first involves an intermediate *in vitro* developmental stage in which ES cells form compact cell structures known as embryoid bodies. These embryoid bodies can induce the developmental program of ES cell differentiation into multiple cell types
20 including cardiomyocytes, which are distinguished in culture by their ability to undergo spontaneous beating. These ES derived *in vitro* differentiated cardiomyocytes recapitulate the normal development of cardiomyocytes as evidenced by the stage-specific expression of cardiomyocyte specific genes. All the known transcription factors, ion channels and structural proteins that are part of normal heart development
25 and function *in vivo* are also expressed in ES-derived cardiomyocytes.

Even though high throughput to medium throughput systems have been developed for functional characterization of cell lines heterologously expressing the gene for specific ion channels, high throughput techniques for functional characterization of more complex systems such as cardiomyocytes have been limited.
30 Technologies designed to assess cardiomyocyte behaviour and function and the effect of drugs and other manipulations *in vitro* can be divided into two different approaches. One approach involves long term assessment of cardiomyocyte viability for example in response to certain compounds. Such assays are typically end point assays designed to measure a cellular component such as ATP which correlates with

the degree of viability of the cells. The other approach involves studying short term effect of drugs and compounds on beating function of cardiomyocytes. High throughput techniques for short term functional characterization of ion channels and other targets in cardiomyocytes has been rather challenging and limited. The available
5 systems typically only monitor a single cardiomyocyte or a small number of cardiomyocytes at a time with very limited throughput.

SUMMARY OF THE INVENTION

10 The invention addresses the need for further study of cardiomyocytes and their response to therapeutic agents by establishing a number beating parameters which may be used to assess the health, function and response of the cardiomyocytes to potential treatment of drugs or chemical compounds. The above is accomplished by providing a method of determining one or more beating parameters for use in
15 cardiomyocyte beating analysis. The method includes providing a cell analysis device including wells, each well including a sensor capable of monitoring beating of cardiomyocytes in millisecond time resolution; adding cardiomyocytes to the wells; monitoring the beating of the cardiomyocytes in millisecond time resolution to obtain a plurality of beating measurements; and calculating one or more beating parameters
20 from the plurality of beating measurements.

The cell analysis device should be able to measure cardiomyocytes beating in millisecond time resolution. In some embodiments, the cell analysis device is an impedance monitoring device, such as an impedance monitoring device having a sensor formed from two electrode structures, each having substantially the same
25 surface area. In other embodiments, the sensor is an optical sensor. Examples of suitable optical sensors are those that can be used to detect or measure changes in cell morphology, cell adhesion and cell number. Among these include a resonant waveguide or a resonant waveguide grating sensor.

In some embodiments, beating measurements are plotted over time to form, at
30 least in part a beating curve. Beating parameters can then be derived from the curve or corresponding data. Among the beating parameters of particular interest, include beating rate, beating amplitude, rising time, falling time, beating period, IBD50, IBD10, IBD90, rising slope, falling slope, normalized beating rate, normalized beating amplitude, beating pattern similarity and beating rhythm irregularity.

Test compounds may be added to identify changes in beating parameters and thus the effect on cardiomyocytes. Accordingly, in some embodiments a test compound is added to the wells at a number of different concentrations and a dose response curve (DRC) is constructed. In further embodiments, the methods include
5 determining an IC50 or EC50 value from the dose response curve. In other embodiments a test compound is added to the wells to assess a potential difference in beating parameters, which may be indicative of cardiotoxicity.

BRIEF DESCRIPTION OF THE DRAWINGS

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FIGS. 1A-B depict schematic drawings of one design of a cell-substrate impedance measurement device of the present invention. **FIG. 1A** shows a nonconductive substrate **101** with 16 electrode arrays or sensors fabricated on the substrate. Each electrode array **102** includes two electrode structures. Each electrode
15 structure comprises multiple electrode elements. Each electrode array connects to two electrical traces **103**, with each of the two traces connected one of the two electrode structures. These electrical connection traces **103** from the electrode array **102** are connected to the connection pads **104** at the edges of the substrate **101**. As shown in **FIG. 1A**, each of the four electrode arrays in each of four quarters on the substrate
20 **101** have one of their electrical connection traces **103** connected to a common connection pad **104**. Thus, for the depicted device there are four common connection pads **104**, one for each quarter of the device. In addition, each electrode array has a separate electrical connection trace **103**, connecting to an independent connection pad **104**. Thus, there are total 20 connection pads **104** at the edges of the substrate (**101**).
25 In **FIG. 1B** a single exemplary electrode array or sensor is shown. The electrode array has two electrode structures, where each electrode structure comprises multiple electrode elements **105** shown here having a circle-on-line geometry. In this electrode array structure, electrode elements **105** of one electrode structure of the array alternate with electrode elements **105** of the other electrode structure of the array. Each of the
30 electrode structures is independently connected to its electrode bus **106**, in this case, by means of direct connection of the electrode elements **105** to the electrode bus **106**. Each electrode bus **106** forms an arc around the perimeter of the array, where the two buses of the array do not abut or overlap. The electrically conductive connection

traces **103** in **FIG. 1A** connect each bus with a connection pad **104** in **FIG. 1A** on the edge of the substrate **101** in **FIG. 1A**.

FIG. 2 depicts graphs demonstrating combined long term and short term measurement of cardiomyocytes beating over at least 7 days including measurement in millisecond time resolution with corresponding cell index values calculated from impedance measurements over beating periods at days 3, 5 and 7.

FIG. 3 depicts graphs showing change in cell index in millisecond time resolution before and after adding 190 nM of compound E4031, which is has pro-arrhythmic effects.

FIG. 4 depicts a series of graphs showing the effect on cell index of a cardiomyocyte cell population in millisecond time resolution before and after adding petamidine, which prevents ERG transport to the membrane and thus a delayed adverse effect over 6 hours.

FIG. 5 depicts a series of graphs showing change in cell index in millisecond time resolution of iPS cell-derived cardiomyocytes and monitoring of the cardiomyocytes over 45 days.

FIG. 6 depicts millisecond time resolution charting of cell index of cardiomyocytes tested with increasing concentration of pro-arrhythmic drugs withdrawn from the US market, namely Droperidol, Astemizole, Cisapride, Sertindole and Terodiline.

FIG. 7 depicts a graphical representation of multiple cardiomyocyte beating periods and graphical illustration of features used in beating parameters including positive peaks P1, P2, P3...Pm; negative peaks -P1, -P2, -P3,...-Pm; and beating amplitude Amp-1, Amp-2, Amp-3...Amp-m.

FIG. 8 depicts a graphical representation of a portion of a beating period to demonstrate a rise and fall of beating amplitude including time points for 20% amplitude, 50% amplitude and 80% amplitude.

FIGS. 9A-D depicts graphs and an accompanying table resulting from dynamic monitoring and characterization of mouse embryonic stem cell derived cardiomyocytes (mESCC) beating using impedance-based detection. **FIG. 9A** shows long term impedance monitoring of cells with arrows depicting time points for short term millisecond recording. **FIG. 9B** shows beating activity and profile of mESCC at time points identified in **FIG. 9A** after cell seeding. The beating rate (1/min), amplitude (delta CI), beat duration (IBD50; ms), Time to Max (ms) and Decay Time

(ms) were quantified using the RTCA Cardio software and as described in the Examples section. The data represents the average of 8 wells $\pm$ Standard Deviation. A total duration of 5 sec recording time is displayed. **FIG. 9C** demonstrates blebbistatin, an inhibitor of myosin heavy chain ATPase activity, inhibits beating activity of mESCC, which is restored by washing out the compound and replacing by normal growth media. **FIG. 9D** demonstrates that Blebibistatin treatment of mESCC has no effect on field potential recording as measured on Multi-Electrode Arrays.

FIGS. 10A-C depict graphs showing pharmacological assessment of ion channel modulators measured using the impedance-based system on mouse embryonic stem cell derived cardiomyocytes (mESCC). Cells were seeded in the wells of the E-PLATE (ACEA Biosciences Inc., San Diego, CA), monitored for 3 days using the RTCA Cardio system and treated with the indicated concentrations of each compound. The beating activity was recorded by the RTCA Cardio system as described in the Examples section. For each compound at the indicated time points 5 sec of beating activity is displayed with the exception of ERG which is total of 14 seconds of beating activity. The beating rate for each interval of beating activity is displayed as beats/min $\pm$ SD; the data shown is one representative recording from a total of at least 3 separate experiments. **FIG. 10A** shows results from treatment with Isradipine, an L-Type voltage-gated calcium channel inhibitor, **FIG. 10B** shows results from treatment with (S)-(-)-Bay K 8644, an agonist of L-Type voltage-gated calcium channels, **FIG. 10C** shows results from treatment with chromanol, inhibitor of the slow delayed rectifier K^+ current.

FIGS. 11A-C depict graphs showing pharmacological assessment of ERG channels, sodium channels and ionotropic agents. **FIG. 11A** shows results from treatment with E4031, an inhibitor of ERG type K^+ channel, **FIG. 11B** shows results from treatment with Tetrodotoxin (TTX), inhibitor of voltage-gated Na^+ channel, and **FIG. 11C** shows results from treatment with isoproterenol, an ionotropic agent and agonist of the β -adrenergic receptor.

FIG. 12 depicts a table summarizing beating parameters including normalized beating rate, normalized amplitude and beating rate irregularity of the compounds tested in **FIGS. 10A-C** and **FIGS. 11A-C**.

FIGS. 13A and B depict graphs showing mechanism-based cardiotoxicity profiling of cardiomyocytes using impedance-based devices. **FIG. 13A** shows

screening, in a dose-response manner in mESCC, 4 drugs which have been withdrawn from the market due to increased incidence of TdP arrhythmia. For each compound a total of 5 sec of beating activity is displayed. For Astemizole, cisapride, droperide and sertindole the dose-response profiles are shown at 30 min, 15 min, 180 min and 165 min after compound addition, respectively. The bottom row shows the dose-response for each of the compounds at the indicated time points based on calculation of beat duration parameter as described in the Examples section. In **FIG. 13B** plateau oscillation profiles are induced by all four compounds tested in **FIG 13A** as well as E-4031, indicating a common underlying mechanism; at total of 9 sec of beating profile recording is displayed for each of the compounds.

FIGS. 14A-C depict graphs and results from treatment with doxorubicin and pentamidine. **FIG. 14A** shows mESCC treated with increasing concentrations of doxorubicin. Dose-dependent impedance-based cellular profiles were monitored for up to 24 hours after compound treatment. In **FIG. 14B** a total of 5 sec of recording is shown for each dose at the given time point. The beating activity is quantified in terms of beating rate and displayed in each box. In **FIG. 14C** mESCC were seeded in the wells of E-PLATE (ACEA Biosciences, San Diego, CA) and on day 3 treated with 20 μ M Pentamidine. The beating activity was monitored at the indicated time windows after compound treatment and quantified based on beat duration as mentioned in the Examples section.

FIG. 15 depicts a table summarizing the screening results of 50 compounds with potential cardiotoxic liability screened at 3 final doses (10 μ M, 1 μ M and 0.1 μ M) using the RTCA Cardio system together with mESCC. For each compound both the lowest observed concentration (LOC) which resulted in an effect on mESCC and the resulting beating profile was included.

DETAILED DESCRIPTION

The present invention addresses the need to provide methods to further improve monitoring of excitation-contraction coupling in cardiomyocytes and excitable cells, such as for cardiac safety assessment and for the study of cardiomyocyte function and differentiation. Specifically, embodiments of the invention describe label-free methods for monitoring cardiomyocytes in vitro and

methods for effective comparison by establishing a plurality of beating parameters. The methods are capable of continuously monitoring excitation-contraction coupling and determining the beating parameters in a relatively high-throughput manner, which offers a unique approach to assessing the safety of potentially cardiotoxic treatments, which may not be readily apparent. The systems and methods can be used to assess both short term and long term effects of substances on cardiomyocyte beating, viability and morphology.

Monitoring beating of cardiomyocytes

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While the invention relates primarily to methods for determining beating parameters for beating analysis and comparison, it is important to obtain reliable beating measurements in millisecond time resolution. Millisecond time resolution preferably refers to the measurement or acquisition of data from at least two consecutive time points within 100 milliseconds of one another. More preferably, the at least two consecutive beating measurements are performed in less than 40 milliseconds. Still more preferably, the at least two consecutive measurements are performed in less than 20 milliseconds. In still further embodiments the at least two consecutive beating measurements are performed in less 10 milliseconds.

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In one approach a device for monitoring the beating of cells may be an optical-based system, which uses an optical sensor. Optical sensors typically measure an optical property of the cell, which may relate cell morphology, cell adhesion degree and/or cell number. Among the optical sensors that may be used include resonant waveguide sensors or resonant wave guide grating sensors. For example, the resonant wave guide grating sensor utilizes the resonant coupling of light into a waveguide through a diffraction grating. A polarized light, covering a range of incident wavelengths, is directly used to illuminate the waveguide; light at specific wavelengths is coupled into and propagates along the waveguide. The resonance wavelength at which a maximum in coupling efficiency is achieved is a function of the local refractive index at or near the sensor surface. When the cardiomyocytes are cultured and attached to the surfaces of the resonant wave guide sensor, the local refractive index on the sensor/cell interface would be affected by changes in cell morphology, cell adhesion and other cellular properties. For example, a relocation or re-arrangement of certain cellular contents such as the change in cell adhesion degree,

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or membrane refilling, recruitment of intracellular proteins to activated receptors at the cell surface, or receptor endocytosis, the change in cell morphology may all result in the change in local refractive index, leading to a detectable change or shift in the maximum in coupling efficiency wavelength. The effect of the presence of the cells
5 on a resonant wave guide grating sensor has been explored based on the dynamic mass redistribution model. The details of such optical sensors and other optical sensors for cell-based assays have been described in detail in "Label-free cell-based assays with optical biosensors in drug discovery", by Ye Fang, in Assay and Drug Development Technologies, Vol 4, pp 583-595, 2006. An optical-sensor based
10 system may include a device comprising optical sensors in wells suitable for cell culture, an optical signal measurement/detection system such as optical CCD camera, an optical-signal processing algorithm to process optical signals in short-time resolution, such as millisecond resolution, to provide a cardiomyocyte-beating dependent curve, and to quantify cell beating and to derive cell-beating parameters
15 (such as calculating average rate of beats per unit time, average amplitude intensity in a unit time as well as the average length of time between the beats) based on cell morphology images.

In the preferred approach the system is an impedance-based cell monitoring system. Most preferably, the system provides a device for monitoring cell-substrate
20 impedance, an impedance analyzer capable of impedance measurements at millisecond time resolution, electronic circuitry that can engage the device and selectively connect two or more sensors or electrode arrays of the device to the impedance analyzer and a software program that controls the electronic circuitry and records and analyzes data obtained from the impedance analyzer. By providing the
25 impedance based system with millisecond time resolution, beating measurements from excitation-contraction coupling of cells can be efficiently monitored in millisecond time resolution. Thus the methods provided herein may be combined with impedance-based systems to identify and evaluate changes in excitation-contraction events and shifts in beating parameters discussed below, which may be
30 used for high throughput analysis of potential therapeutics. The skilled artisan will appreciate that millisecond time resolution measurements may be coupled with longer term impedance monitoring, such as longer than seconds, hours or days. In some embodiments, long term impedance monitoring is performed with intermittent periods of impedance monitoring at millisecond time resolution. Suitable impedance

monitoring systems are described in detail in US patent application serial no. 12/435,569, which is herein incorporated by reference in its entirety. A preferred electrode array or configuration is also provided in **FIGS. 1A** and **B**.

5 The preferred device for monitoring substrate impedance includes a nonconductive substrate having one or more individually addressable electrode arrays or sensors fabricated thereon and one or more wells. A surface of the substrate may be suitable for cell attachment, wherein the cell attachment results in a detectable change in impedance between electrodes within the array. Preferably, the nonconducting substrate is planar, and is flat or approximately flat. The substrates
10 may be constructed from a variety of nonconductive materials known in the present art, including, but not limited to, silicon dioxide on silicon, silicon-on-insulator (SOI) wafer, glass (e.g., quartz glass, lead glass or borosilicate glass), sapphire, ceramics, polymer, fiber glass, plastics, *e.g.*, polyimide (e.g. Kapton, polyimide film supplied by DuPont), polystyrene, polycarbonate, polyvinyl chloride, polyester, polypropylene
15 and urea resin. Preferably, the substrate is biocompatible with excitable cells such as cardiomyocytes; however, materials that are not biocompatible can be made biocompatible by coating with a suitable material, such as a biocompatible polymer or coating. Further, attachment or growth along the substrate or electrodes may be enhanced by pre-coating the substrate with a protein or compound that facilitates
20 attachment or growth. Such compounds may be chosen according to techniques known in the cellular biology arts; however, in some embodiments fibronectin is effective. Alternatively, the substrate may be chemically modified to display reactive groups that enhance cell attachment, particularly ES cells or cardiomyocytes.

Preferably, each sensor or electrode array includes two or more electrode
25 structures that are constructed to have dimensions and spacing such that they can, when connected to a signal source, operate as a unit to generate an electrical field in the region of spaces around the electrode structures. Preferably the electric field is substantially uniform across the array. An electrode structure refers to a single electrode, particularly one with a complex structure. Specifically, an electrode array
30 includes two electrode structures, each of which includes multiple electrode elements, or substructures, which branch from the electrode structure. In preferred embodiments, the electrode structures of each of the two or more electrode arrays of a device have substantially the same surface area.

Each of the two electrode structures of an electrode array is connected to a separate connection pad that is preferably located at the edge of the substrate. Specifically, for each of the two or more electrode arrays of the device, preferably the first of the two electrode structures is connected to one of the two or more connection pads, and the second of the two electrode structures is connected to another of the two or more connection pads. Preferably, each array of a device is individually addressed, meaning that the electrical traces and connection pads of the arrays are configured such that an array can be connected to an impedance analyzer in such a way that a measuring voltage can be applied across a single array at a given time using switches (such as electronic switches).

Preferably, each electrode array of the device has an approximately uniform electrode resistance distribution across the entire array. By "uniform resistance distribution across the array" is meant that when a measurement voltage is applied across the electrode structures of the array, the electrode resistance at any given location of the array is approximately equal to the electrode resistance at any other location on the array. Preferably, the electrode resistance at a first location on an array of the device and the electrode resistance at a second location on the same array do not differ by more than 30%. More preferably, the electrode resistance at a first location on an array of the device and the electrode resistance at a second location on the same array do not differ by more than 15%. Even more preferably, the electrode resistance at a first location on an array of the device and a second location on the same array do not differ by more than 5%. More preferably yet, the electrode resistance at a first location on an array of the device and a second location on the same array do not differ by more than 2%.

Preferred arrangements for electrode elements and gaps between the electrodes and electrode buses in a given electrode array are used to allow all cells, no matter where they land and attach to the electrode surfaces and to contribute similarly to the total impedance change measured for the electrode array. Thus, it is desirable to have similar electric field strengths at any two locations within any given array of the device when a measurement voltage is applied to the electrode array. At any given location of the array, the field strength is related to the potential difference between the nearest point on a first electrode structure of the array and the nearest point on a second electrode structure of the array. It is therefore desirable to have similar electric potential drops across the electrode elements and across the electrode buses of a given

array. Based on this requirement, it is preferred to have an approximately uniform electrode resistance distribution across the whole array where the electrode resistance at a location of interest is equal to the sum of the electrode resistance between the nearest point on a first electrode structure (that is the point on the first electrode structure nearest the location of interest) and a first connection pad connected to the first electrode structure and the electrode resistance between the nearest point on a second electrode structure (that is the point on the first electrode structure nearest the location of interest) and a second connection pad connected to the second electrode structure.

Preferably, devices of the present invention are designed such that the arrays of the device have an approximately uniform distribution across the whole array. This can be achieved, for example, by having electrode structures and electrode buses of particular spacing and dimensions (lengths, widths, thicknesses and geometrical shapes) such that the resistance at any single location on the array is approximately equal to the resistance at any single other location on the array. In most embodiments, the electrode elements (or electrode structures) of a given array will have even spacing and be of similar thicknesses and widths, the electrode buses of a given array will be of similar thicknesses and widths, and the electrode traces leading from a given array to a connection pad will be of closely similar thicknesses and widths. Thus, in these preferred embodiments, an array is designed such that the lengths and geometrical shapes of electrode elements or structures, the lengths and geometrical shapes of electrode traces, and the lengths and geometrical shapes of buses allow for approximately uniform electrode resistance distribution across the array.

In some preferred embodiments of impedance measurement devices, electrode structures comprise multiple electrode elements, and each electrode element connects directly to an electrode bus. Electrode elements of a first electrode structure connect to a first electrode bus, and electrode elements of a second electrode structure connect to a second electrode bus. In these embodiments, each of the two electrode buses connects to a separate connection pad via an electrical trace. Although the resistances of the traces contribute to the resistance at a location on the array, for any two locations on the array the trace connections from the first bus to a first connection pad and from the second bus to a second connection pad are identical. Thus, in these preferred embodiments trace resistances do not need to be taken into account in

designing the geometry of the array to provide for uniform resistances across the array.

In preferred embodiments of the present invention, a device for monitoring cell-substrate impedance has two or more electrode arrays that share a connection pad. Preferably one of the electrode structures of at least one of the electrode arrays of the device is connected to a connection pad that also connects to an electrode structure of at least one other of the electrode arrays of the device. Preferably for at least two arrays of the device, each of the two or more arrays has a first electrode structure connected to a connection pad that connects with an electrode structure of at least one other electrode array, and each of the two or more arrays has a second electrode structure that connects to a connection pad that does not connect with any other electrode structures or arrays of the device. Thus, in preferred designs of a device there are at least two electrode arrays each of which has a first electrode structure that is connected to a common connection pad and a second electrode structure that is connected to an independent connection pad.

In some preferred embodiments of the present invention, each of the electrode structures of an array is connected to an electrode bus that is connected to one of the two or more connection pads of the device via an electrically conductive trace. In preferred embodiments, each of the two electrode structures is connected to a single bus, such that each array connects to two buses, one for each electrode structure. In this arrangement, each of the two buses connects to a separate connection pad of the substrate.

The electrically conductive traces that connect a bus with a connection can be fabricated of any electrically conductive material. The traces can be localized to the surface of the substrate, and can be optionally covered with an insulating layer. Alternatively the traces can be disposed in a second plane of the substrate. Description of arrangements and design of electrically conductive traces on impedance measurement devices can be found in U.S. Patent No. 7,470,533, herein incorporated by reference for all disclosure on fabrication and design of electrically conductive trace on substrates.

Appropriate electronic connection means such as metal clips engaged onto the connection pads on the substrate and connected printed-circuit-boards can be used for leading the electronic connections from the connection pads on the devices to external electronic circuitry (e.g. an impedance analyzer). Description of the design of cell-

substrate impedance devices and their manufacture can be found in U.S. Patent 7,470,533, herein incorporated by reference for all description and disclosure of the design, features, and manufacture of impedance device comprising electrode arrays.

5 Descriptions of electrode arrays used for impedance measurement that apply to the devices of the present invention are also described in U.S. Patent No. 7,470,533, herein incorporated by reference for all disclosure relating to electrode arrays (or structural units), electrode structures, electrode materials, electrode dimensions, and methods of manufacturing electrodes on substrates.

10 Preferred electrode arrays for devices of the present invention include arrays comprising two electrode structures, such as, for example, spiral electrode arrays and interdigitated arrays. In some preferred devices of the present invention, electrode arrays are fabricated on a substrate, in which the arrays comprises two electrode structures, each of which comprises multiple circle-on-line electrode elements, in which the electrode elements of one structure alternate with the electrode elements of
15 the opposite electrode structure. Electrode arrays may be provided in configurations, such as interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal and castellated.

Preferably, the electrode elements (or electrode structures) of an array of the present device of the present invention are of approximately equal widths. Preferably
20 the electrode elements (or electrode structures) of an array of the present device of the present invention are greater than 20 microns and less than 500 microns in width, more preferably from about 50 to about 300 microns in width.

Preferably, the electrode elements (or electrode structures) of an array of the present device of the present invention are approximately evenly spaced. Preferably,
25 the gap between electrode elements (or electrode structures) of an array of the present device of the present invention is less than 100 microns and more than 5 microns in width, more preferably from about 10 to about 80 microns in width.

Preferably, the device includes one or more fluid-impermeable receptacles which serve as fluid containers or wells. Such receptacles may be reversibly or
30 irreversibly attached to or formed within the substrate or portions thereof (such as, for example, wells formed as in a microtiter plate). In another example, the device of the present invention includes microelectrode strips reversibly or irreversibly attached to plastic housings that have openings that correspond to electrode structure units located on the microelectrode strips. Suitable fluid container materials comprise

plastic, glass, or plastic coated materials such as a ceramic, glass, metal, etc. Descriptions and disclosure of devices that comprise fluid containers can be found in U.S. Patent No. 7,470,533, herein incorporated by reference for all disclosure of fluid containers and fluid container structures that can engage a substrate comprising electrodes for impedance measurements, including their dimensions, design, composition, and methods of manufacture.

In preferred embodiments, each electrode array on the substrate of a device of the present invention is associated with a fluid-impermeable container or receptacle, such as, for example, a well. Preferably, the device of the present invention is assembled to a bottomless, multiwell plastic plate or strip with a fluid tight seal. The device is assembled such that a single array of the substrate is at the bottom of a receptacle or well. Preferably, each array of a device is associated with a well of a multiwell plate. In some preferred embodiments, a multiwell device for cell-substrate impedance measurement has "non-array" wells that are attached to the substrate but not associated with arrays. Such wells can optionally be used for performing non-impedance based assays, or for viewing cells microscopically.

The design and assembly of multiwell impedance measurement devices is described in U.S. Patent No. 7,470,533, and also in U.S. Patent No. 7,192,752, both herein incorporated by reference for disclosure of multiwell impedance measurement devices, including their design, composition, and manufacture. A device of the present invention preferably has between 2 and 1,536 wells and more preferably between 4 and 384 wells. In some embodiments the device includes 6 wells, 16 wells, 32 wells, 96 wells or 386 wells.

In some preferred embodiments, commercial tissue culture plates can be adapted to fit a device of the present invention. Bottomless plates may also be custom-made to preferred dimensions. Preferably, well diameters are from about 1 millimeter to about 20 millimeters, more preferably from about 2 millimeters to about 8 millimeters at the bottom of the well (the end disposed on the substrate). The wells can have a uniform diameter or can taper toward the bottom so that the diameter of the container at the end in contact with the substrate is smaller than the diameter of the opposing end.

In the system for monitoring impedance of beating cells the impedance analyzer engages connection pads of one or more multi-well devices to measure impedance. In one embodiment of the above system, the impedance analyzer is

capable of measuring impedance between 0.1 ohm and 10^5 ohm in frequency range of 1 Hz to 1 MHz. The impedance analyzer is preferably capable of measuring both resistance and reactance (capacitive reactance and inductive reactance) components of the impedance. In a preferred embodiment of the above system, the impedance
5 analyzer is capable of measuring impedance between 1 ohm and 10^3 ohm in frequency range of 1.00 Hz to 300 kHz.

In preferred embodiments the impedance analyzer is capable of impedance measurements at millisecond time resolution. The required or desired time resolution may vary depending on the excitation cycle of the excitable cell. Excitable cells
10 having shorter excitation cycles would tend to require faster time resolution. In some embodiments 500 millisecond time resolution is sufficient, such that at least two consecutive impedance measurements are between about 300 milliseconds and about 500 milliseconds apart. In preferred embodiments, impedance measurement at millisecond time resolution includes at least two consecutive impedance
15 measurements less than 100 milliseconds apart. In some instances the at least two consecutive impedance measurements are less than 50 milliseconds or less than 40 milliseconds apart. In some instances the at least two consecutive impedance measurements are less than 20 milliseconds apart. In some instances at least two consecutive impedance measurements are less than 10 milliseconds apart. In some
20 instances millisecond time resolution includes two consecutive impedance measurements between 1 millisecond and 5 milliseconds, between 5 milliseconds and 10 milliseconds, between 10 milliseconds and 20 milliseconds, between 20 milliseconds and 40 milliseconds, or between 40 milliseconds and 50 milliseconds apart. In some instances millisecond time resolution includes at least two consecutive
25 impedance measurements between 50 milliseconds and 100 milliseconds apart. In some instances millisecond time resolution includes at least two consecutive impedance measurements between 100 milliseconds and 150 milliseconds or between 150 and 300 milliseconds apart.

With millisecond time resolution for impedance measurement, it becomes
30 possible to resolve individual beating cycles of cardiomyocytes cultured on electrodes. Whilst theoretically one needs at least two data points for each beating cycle, in practice more than 2 data points are needed for each beating cycle. For example, if cells have a beating rate of 60 beats per minute, i.e., one beat per second. It would be preferred to have a time resolution of at least 200 milliseconds so that

each beating cycle consists of 5 data points. More preferably, the measurement time resolution is 100 milliseconds. Still more preferably, the time resolution is 50 milliseconds or less.

One skilled in the art will understand that the cell-substrate impedance measurement or monitoring system with millisecond time resolution can be used to efficiently and simultaneously perform multiple assays by using circuitry of the device station to digitally switch from recording from measuring impedance over an array in one well to measuring impedance over an array in another well. Similarly, groups of wells may be monitored simultaneously and switched between designated groups. In one embodiment of the above system, the system under software control is capable of completing an impedance measurement for an individual well at a single frequency within milliseconds, such as less than 100 milliseconds, less than 40 milliseconds, less than 20 milliseconds, less than 10 milliseconds or between 1 millisecond and 40 milliseconds. In some embodiments the user may choose the frequency of measurement for millisecond time resolution.

A multiple-well cell-substrate impedance measuring device in a system of the present invention can be any multiple-well cell-substrate impedance measuring device in which at least two of the multiple wells comprise an electrode array at the bottom of the well, and in which at least two of the multiple wells comprise an electrode array are individually addressed. In one embodiment of the above system, the multi-well device takes the form of a specialized microtiter plate which has microelectronic sensor arrays integrated into the bottom of the wells.

A device used in a system of the present invention, when connected to an impedance analyzer, can measure differences in impedance values that relate to cell behavior. For example, a cell-substrate impedance measuring device used in a system of the present invention can measure differences in impedance values when cells are attached to the electrode array and when cells are not attached to the electrode array, or can detect differences in impedance values when the number, type, activity, adhesiveness, or morphology of cells attached to the electrode-comprising surface of the apparatus changes. Further, by using millisecond time resolution differences in impedance may be detected or monitored that relate to excitation-contraction coupling, including the beating of cardiomyocytes or stem cells differentiating into cardiomyocytes, and the signaling between neurological cells. Impedance monitoring of the excitation cycle of excitable cells may be determined and monitored before,

during or after adding a test compound, which is suspected of affecting the excitation cycle. Thus, by monitoring the excitation cycle of the excitable cell before, during or after adding a test compound the system provides data corresponding to the potential affect of the compound on the cardiovascular system, the heart, the nervous system, and the like. In some embodiments monitoring the excitation cycle of the cell before, during or after adding a compound provides cardiotoxicity data useful in drug screening.

In some embodiments a device station or electromechanical apparatus or assembly capable of interfacing multiwell devices can include one or more platforms or one or more slots for positioning one or more multiwell devices. The one or more platforms or one or more slots can comprise sockets, pins or other devices for electrically connecting the device to the device station. The device station or electromechanical apparatus or assembly capable of interfacing multiwell devices preferably can be positioned in a tissue culture incubator during cell impedance measurement assays. It can be electrically connected to an impedance analyzer and computer that are preferably located outside the tissue culture incubator.

The device station or electromechanical apparatus or assembly capable of interfacing multiwell devices includes electronic circuitry that can connect to the impedance monitoring device and an impedance analyzer and electronic switches that can switch on and off connections to each of the two or more electrode arrays of the multiwell devices used in the system. The switches of the device station or electromechanical apparatus or assembly capable of interfacing multiwell devices are controlled by a software program, each of which has been improved to provide millisecond time resolution. The software program directs the device station to connect arrays of the device to an impedance analyzer and monitor impedance from one or more of the electrode arrays. During impedance monitoring, the impedance analyzer can monitor impedance at one frequency or at more than one frequency. Preferably, impedance monitoring is performed at more than one time point for a given assay, and preferably, impedance is monitored using at least two time points. The device station can connect individual arrays of a device to an impedance analyzer to monitor one, some, or all of the arrays of a device for a measurement time point. In some preferred embodiments of the present invention, the device station software is programmable to direct impedance monitoring of any of the wells of the device that comprise arrays at chosen time intervals.

The software of the impedance monitoring system can also store and display data. Data can be displayed on a screen, as printed data, or both. Preferably the software can allow entry and display of experimental parameters, such as descriptive information including cells types, compound concentrations, time intervals monitored, etc. Further, since a plurality of beating parameters are obtained using the beating measurements, software provides menus to select one or more of the beating parameters for analysis.

The software, termed RTCA CARDIO SOFTWARE (ACEA Biosciences Inc., San Diego, CA), also permits fast analysis of beating parameters. Thus, after obtaining impedance measurements the software can calculate or determine from the impedance measurement a plurality of beating parameters such as beating rate, beating amplitude, rising time, falling time, beating period, IBD10, IBD50, IBD90, rising slope, falling slope, normalized beating rate, normalized beating amplitude, beating pattern similarity and beating rhythm irregularity, and perform subsequent statistics, such as average and standard deviation and further supply IC50 or EC50 values for dose-response testing.

Determining cell index from impedance or optical measurements

Although raw impedance values or raw values obtained from optical sensors may be used as beating measurements in preferred embodiments the raw impedance values are converted to cell index values or delta cell index values for comparison or further derivation into beating parameters as discussed below. Information regarding how to calculate a cell index, cell change index, normalized cell index, and delta cell index may be found in US patent application serial no. 12/435,569, US patent application serial no. 11/903,454, and U.S. Patent No. 7,470,533, the contents of which are herein incorporated by reference with respect to the cell index, cell index number, cell change index, and cell change index number. However a briefly summary is provided.

The cell index obtained for a given well reflects: 1) how many cells are attached to the electrode surfaces in this well, and 2) how well cells are attached to the electrode surfaces in the well. In this case, a zero or near-zero "cell index or cell number index" indicates that no cells or very small number of cells are present on or attached to the electrode surfaces. In other words, if no cells are present on the

electrodes, or if the cells are not well-attached onto the electrodes the cell index equals 0. A higher value of “cell index” or “cell number index” indicates that, for the same type of the cells and cells under similar physiological conditions, more cells are attached to the electrode surfaces. Thus cell index is a quantitative measure of cell number present in a well. A higher value of “cell index” may also indicate that, for the same type of the cells and the same number of the cells, cells are attached better (for example, cells spread out more, or cell adhesion to the electrode surfaces is stronger) on the electrode surfaces.

Non-limiting examples for determining cell index follow. Cell index can be calculated by: at each measured frequency, calculating the resistance ratio by dividing the measured resistance (when cells are attached to the electrodes) by the baseline resistance; finding or determining the maximum value in the resistance ratio over the frequency spectrum; and subtracting one from the maximum value in the resistance ratio. In another variation, cell index is determined by: at each measured frequency, calculating the resistance ratio by dividing the measured resistance (when cells are attached to the electrodes) to the baseline resistance; finding or determining the maximum value in the resistance ratio over the frequency spectrum; and taking a log-value (e.g., based on 10 or $e=2.718$) of the maximum value in the resistance ratio. In another variation, cell index is determined by: at each measured frequency, calculating the reactance ratio by dividing the measured reactance (when cells are attached to the electrodes) to the baseline reactance; finding or determining the maximum value in the reactance ratio over the frequency spectrum; and subtracting one from the maximum value in the reactance ratio. In still another variation, cell index can be calculated by: at each measured frequency, calculating the resistance ratio by dividing the measured resistance (when cells are attached to the electrodes) to the baseline resistance; then calculating the relative change in resistance in each measured frequency by subtracting one from the resistance ratio; and then integrating all the relative-change value.

It is worthwhile to point out that it is not necessary to derive such a “cell index” for utilizing the impedance information for monitoring cell conditions over the electrodes. Actually, one may choose to directly use impedance values (e.g., at a single fixed frequency; or at a maximum relative-change frequency, or at multiple frequencies) as an indicator of cell conditions.

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. Normalized cell index is cell index normalized against cell index at a particular time point. In most cases in the present applications, normalized cell index is derived as normalized relative to the time point immediately before a compound addition or treatment. Thus, normalized cell index at such time point (immediately before compound addition) is always unit one for all wells. One possible benefit for using such normalized cell index is to remove the effect from difference in cell number in different wells. A well having more cells may produce a larger impedance response following compound treatment. Using normalized cell index, it helps to remove such variations caused by different cell numbers.

A “delta cell index” at a given time point is calculated by subtracting the cell index at a standard time point from the cell index at the given time point. Thus, the delta cell index is the absolute change in the cell index from an initial time (the standard time point) to the measurement time.

The time-dependent cellular response (including cardiotoxicity response) may be analyzed by deriving parameters that directly reflect the changes in cell status. For example, time dependent cellular response may be analyzed by calculating the slope of change in the measured impedance responses (that is equivalent to the first order derivative of the impedance response with respect to time, impedance response here can be measured impedance data or derived values such as cell index, normalized cell index or delta cell index). In another example, the time-dependent cellular responses (including cardiotoxic responses) may be analyzed for their higher order derivatives with respect to time. Such high order derivatives may provide additional information as for how cells responding to different compounds and as for the mechanisms of compound action.

The use of cell index together with millisecond time resolution impedance monitoring is demonstrated in **FIGS. 2-6**, which depict high resolution images of the cardiomyocyte beating cycle and pattern shifting in response to administration of cardiotoxic compounds. While cell index permits graphically depicting the beating cycle, still more detailed analysis of beating parameters reveals improvements to cardiomyocyte population analysis.

Beating parameters derived from millisecond time resolution measurements or cell index

While impedance measurements, optical measurements and cell index values
5 can provide valuable information about a cell population, a variety of beating
parameters are established which further assist in the analysis of a beating
cardiomyocyte, a beating cell or cell population differentiating into a beating cell
population. Further, the beating parameters permit comparisons before and after
treatment with a compound, typically a test compound, to assess its effect or predicted
10 effect on a cardiomyocyte cell population and the like. As such, the beating
parameters may be used to establish or confirm safety of a compound or provide
further insight as to the potential mechanism of action of a compound, such as its
effect on stem cell differentiation to or away from a cardiomyocyte cell type or
characteristic. Further, by providing a compound at various concentrations its dose
15 response can be studied. The below beating parameters have been found useful in
assessing potential effects on cardiomyocytes and thus each alone or in combination
can be used to assess potential risk of compound based therapies.

In one approach beating measurements are used to determine beating cycle
peaks associated with a cell. Beating itself corresponds to the excitation-contraction
20 coupling of the cells. Turning to **FIG. 7**, beatings are defined as a sequence of
Positive Peaks (+P as labeled) and Negative Peaks (-P as labeled). The value of these
Positive Peaks and Negative Peaks and the corresponding time periods determine the
beating characteristics, which reveal the status of the cardiomyocyte population. For
example, in **FIG. 7**, a Positive Peak may correspond to the contraction of
25 cardiomyocytes, whilst the return of measurement values to baseline and to negative
peak may correspond to the relaxation of cardiomyocytes.

As an example, time dependent impedance values or cell index values for a
well are analyzed by deriving their first order derivatives and second order derivatives
using numerical methods. The beating cycle peaks are those data points where the
30 first order derivatives of impedance values or cell index values are zero or close to
zero in absolute value. If the beating cycle peak is a positive peak (i.e. peak
corresponds to a maximum value in measured impedance or cell index over the
beating cycle), then the peak would correspond to data points where the second order
derivatives of the impedance values or cell index values are negative and where the

first order derivatives of the impedance values or cell index values are zero or close to zero in absolute value. If the beating cycle peak is a negative peak (i.e. peak corresponds to a minimum value in measured impedance or cell index over the beating cycle), then the peak would correspond to the data points where the second order derivatives of the impedance values or cell index values are positive and where the first order derivatives of the impedance values or cell index values are zero or close to zero in absolute value.

In yet another approach, the method for searching for and identifying “positive peaks” and “negative peaks” may involve the use and modification of various mathematical algorithms, e.g., the Douglas–Peucker algorithm. The Douglas–Peucker algorithm is an algorithm for reducing the number of points in a curve that is approximated by a series of points. Based on the required maximum distance between on the original curves and on the simplified curves, the Douglas–Peucker algorithm could also be adopted to identify positive peaks and negative peaks in time-dependent data point series for impedance values and/or cell index values.

In another approach, a method of determining a beating cycle peak is to search for the data point where the trend of the data changes direction from “increasing” to “decreasing” with time (for a positive peak), or from “decreasing” to “increasing” (for a negative peak). After the identification of the beating cycle peaks, the impedance or cell index values at such peak time points provide the magnitude or amplitude of the beating cycle peaks.

After determining the beating cycle peaks, various methods can be used to calculate the beating rate. A beating rate parameter is generally provided as beatings per minute. In a positive peak counting approach, the number of positive peaks is determined over a given time interval and converted to the desired unit, preferably beats per minute. Similarly, in a negative peak counting approach the number of negative peaks is determined over given time interval and converted to the desired unit. As an example, if there are 2 peaks in a one second interval, then the beating rate would be 2 beats per second, or 120 beats per minute. In still another approach, beating rate is calculated by determining the time period between a series of two or more positive peaks or between a series of two or more negative peaks. That is, in this approach a unit of time (e.g., 1 minute) is divided by the time period between two adjacent peaks. For example, if two adjacent peaks are separated by 500 milliseconds, then the beating rate would be 120 beats per minute. In a time interval

comprising multiple positive or negative peaks, the beating rate could be determined by the following method. Take positive peaks as an example, the time periods between every pairs of two adjacent positive peaks are calculated. Then the beating rate could be calculated in two ways. The first method is to divide a unit of time (e.g., 1 minute) by the average of the time periods between all two-adjacent positive peaks in the given time interval. The second method is to calculate the corresponding beating rates based on each pair of two adjacent positive peaks and then to average of the adjacent-peaks-derived beating rates.

To further assist in comparison, beating rates can also be normalized. Determining a normalized beating rate is achieved by dividing the beating rate at a selected data analysis time by a beating rate at a normalization time. Thus a beating rate identical to that at the normalization time would be defined as 1. Normalizing beating rates can provide a more clear indication of whether and to what degree a change in beating rate occurs. For example, normalization time corresponds to the time point of measurement immediately prior to a compound treatment of the cardiomyocytes. Thus, normalized beating rates could provide clear information as for what the effect of the compound has on the beating rates. A normalized beating rate close to 1 or equal to 1 means that the compound does not have much effect on the beating rate, A normalized beating rate smaller than 1 means that the compound may result in reduction in the beating rates of cardiomyocytes. A normalized beating rate larger than 1 means that the compound may cause an increase in the beating rates of cardiomyocytes. Normalized beating rates could be derived for beating rates calculated using different methods such as peak-counting derived beating rates and beating peak periods derived beating rates.

Beating amplitude is a parameter used in some embodiments to describe or correspond to the intensity of the peak, which may reflect the extent of contraction or relaxation of cardiomyocytes during a beating cycle. Determining beating amplitude can involve a whole peak approach, which could be determined by the difference between a negative peak and the following positive peak as shown in **FIG. 7**. For example, in **FIG. 7** the beating amplitude is shown as the difference in cell index between a Negative Peak to the following adjacent Positive Peak (Amp-1, Amp-2, Amp-3, ..., Amp-m). In another approach, beating amplitude for a positive peak is the difference between a determined baseline to a positive peak. In still another

approach beating amplitude for a negative peak is the difference between a determined baseline to a negative peak. An exemplary baseline is shown in **FIG. 7**.

Thus, for a single beating cycle, one could define or identify different types of amplitude (or an amplitude) of the beating-cycle peaks, including the amplitude of positive peak, the amplitude of negative peak and the amplitude of the whole peak in a cycle. From the measured data point series, a baseline value, which may theoretically correspond to the value when the cardiomyocytes are at their fully relaxation status, could be determined or identified from the measured data values in a time series. The amplitude of a positive peak is the impedance value or cell index value or other measurement value at the positive-peak time point subtracted by the baseline value. The amplitude of a negative peak is the impedance value or cell index value or other measurement value at the negative-peak time point subtracted by the baseline value. The amplitude of whole peak is the difference in the impedance value or cell index value or other measurement value between positive-peak time point and negative-peak time point.

Whilst the above paragraph discusses different types of the amplitudes of a single beating cycle, for a time period including multiple beating cycles, one could determine the average and standard deviations (or standard errors) of the positive-peak amplitude, the negative-peak amplitude and whole-peak amplitude.

Beating amplitude can also be normalized as a normalized amplitude. A normalized amplitude is the amplitude at a selected data analysis time divided by the amplitude at the normalization time point. A beating amplitude identical to that at the normalization time would be defined as 1. Thus, the normalized amplitude reveals differences, such as an increase or decrease in the amplitude or intensity of a beat compared to a referenced amplitude. For example, normalization time corresponds to the time point of measurement immediately prior to a compound treatment of the cardiomyocytes. Thus, normalized beating amplitudes could provide clear information as for what the effect of the compound has on the beating amplitudes. A normalized beating amplitude close to 1 or equal to 1 means that the compound does not have much effect on the beating amplitude. A normalized beating amplitude smaller than 1 means that the compound may result in reduction in the beating amplitudes of cardiomyocytes. A normalized beating amplitude larger than 1 means that the compound may cause an increase in the beating rates of cardiomyocytes.

Normalized beating amplitude could be derived for all three types of beating amplitudes, i.e. positive-peak based amplitude, negative-peak based amplitude and whole-peak amplitude.

In some instances, it is more useful to consider a beating amplitude that is less than the difference between the positive peak and negative peak or baseline. For example, it may be desirable to consider a portion of the amplitude, such as a 10% amplitude, 20% amplitude, 50% amplitude, 80% amplitude, 90% amplitude and the like. This may be particularly preferred when assessing other parameters together with beating amplitude such as when considering a rising slope or an IBD10, IBD50, IBD90 and the like.

Rising time T_r is a parameter which provides the amount of time to travel the rising slope between the negative peak and the positive peak (or between the baseline and the positive peak). Typically, rising time is provided as an average over many beatings and is converted to time in the unit of seconds for analysis or comparison. In some embodiments rising time is calculated as the time needed to reach a positive peak or maximum amplitude from a negative peak or a baseline. Referring to **FIG. 8** as an example, in other instances, rising time T_r is calculated between data points positioned below the positive peak and above the negative peak (or below the positive peak and above the baseline). That is, since the slope of the beating curve changes dramatically at the peaks, it is often preferred to consider the rising time between 20% amplitude and 80% amplitude for comparison. Thus, when determining rising time it may be necessary to first determine the beating amplitude then determine suitable comparison points such as 20% amplitude, 50% amplitude, 80% amplitude and the like. By one example as illustrated in **FIG. 8**, rising slope can be calculated by the dividing the cell index change from 20% amplitude to 80% amplitude by the time it takes as follows:

$$\text{Rising Slope} = (\text{Amp}_{80} - \text{Amp}_{20}) / T_r$$

Depending on specific application, it is possible that we may define other rising time and/or rising slopes, using different percentage amplitude points. For example, rising slope may be defined as:

$$\text{Rising Slope} = (\text{Amp}_{90} - \text{Amp}_{10}) / T_r$$

Falling time T_f is a beating parameter which provides the amount of time to travel the falling slope between a positive peak or a point of maximum amplitude to a negative peak or a baseline. Falling time T_f can be provided as an average over many
5 beatings and is generally converted to time in the unit of seconds for analysis or comparison. In some embodiments falling time T_f is calculated as the time needed to reach a negative peak or baseline from a positive peak; however, referring again to **FIG. 8** as an example, in other instances, falling time T_f is calculated between data points positioned below the positive peak and above the negative peak. That is, since
10 the slope of the beating curve changes dramatically at the peaks, it is often preferred to consider the falling time between 80% amplitude (or 90% amplitude) and 20% amplitude (or 10% amplitude) for comparison. Thus, when determining falling time it may be necessary to first determine the beating amplitude then determine suitable comparison points such as 10% amplitude, 20% amplitude, 50% amplitude, 80%
15 amplitude, 90% amplitude and the like. By way of example, falling slope can be calculated by dividing the cell index change from 80% amplitude to 20% amplitude by the time it takes as follows:

$$\text{Falling Slope} = (\text{Amp}_{80} - \text{Amp}_{20}) / T_f$$

20

Depending on specific application, it is possible that we may define other rising time and/or rising slopes, using different percentage amplitude points. For example, falling slope may be defined as:

25

$$\text{Falling Slope} = (\text{Amp}_{90} - \text{Amp}_{10}) / T_f$$

30

IBD50 provides the time window between time points where the beating signals attains 50% amplitude. IBD50 is particularly useful when studying the effect of the compounds on the beating cycle of cardiomyocytes. For example, if a compound, through various mechanisms, results in prolongation of contraction phase of the beating cycle of the cardiomyocytes, then IBD50 derived from the beating cycle waveform may be increased as a result of the treatment of cardiomyocytes with this compound. In another example, if a compound results in certain pro-arrhythmia effects in cardiomyocytes (see **FIG. 3** as an example where E4031 induced

arrhythmia effects), then IBD50 derived from the beating cycle waveform may also be significantly altered. Similarly, other time windows can be used instead to assess similar pro-arrhythmia effects at different percentage of the amplitude such as 10% amplitude and 90% amplitude to give IBD10 and IBD90. Time between following
 5 IBD10, IBD50 or IBD90 measurements are designated as T_{IBD10} , T_{IBD50} , and T_{IBD90} . An exemplary T_{IBD50} parameter is shown in **FIG. 8**.

Beating period (also referred to as “beating cycle”) is a parameter which provides the time period between two positive peaks, two negative peaks or can be the time period between a positive peak and a negative peak. The beating period can be
 10 used to identify changes in beating rate or can be used as a defined period for comparison of other parameters, such as differences in amplitude, rising time, falling time and the like. In **FIG. 7**, within one Sweep Duration, the number of Positive Peaks is m ($+P1$, $+P2$, $+P3$, ..., $+Pm$) and the number of Negative Peaks is n ($-P1$, $-P2$, $-P3$, ..., $-Pn$). The time between two adjacent individual Positive Peaks (or/and
 15 two adjacent individual Negative Peaks) is defined as beating period. For example, the beating period based on the Positive Peaks is T_{+P1} , T_{+P2} , ..., $T_{+P(m-1)}$ and the beating period based on the Negative Peaks is T_{-P1} , T_{-P2} , ..., $T_{-P(n-1)}$.

Rising slope is a parameter corresponding to the slope that occurs during a specified time period when the measured impedance values or cell index values or
 20 other measurement/derived values increase with time. That is, the rising slope is a slope between a negative peak and an adjacent baseline point (which is after the negative peak in time), or between certain baseline point and an adjacent positive peak (which is after the baseline point in time), or between a negative peak and its immediately adjacent positive peak (which is after the negative peak in time). For a
 25 single beat, one could determine rising slopes based on different definitions as described in the previous sentence. The rising slope may be an average between multiple slopes across multiple beats.

Falling slope is a parameter that corresponds to the slope that occurs during a specified time period when the measured impedance values or cell index values or
 30 other measurement/derived values decrease with time. Thus, the falling slope is a slope between a positive and an adjacent baseline point (which is after the positive peak in time), or between certain baseline point and a negative peak (which is after the baseline point in time), or between a positive peak and its immediately adjacent negative peak (which is after the positive peak in time). For a single beat, one could

determine falling slopes based on different definitions as described in the previous sentence. The falling slope may be an average between multiple slopes across multiple beats.

Beating pattern similarity is a parameter derived to quantify the degree of the similarity between the beating waveforms between two different time intervals. For any given time interval, the beating pattern is shown as the beating curves comprised of a number of measurement values (impedance values, cell index values or other values) across a number of time points during the time interval. Beating patterns at two time intervals may be compared numerically, such as by comparison between determined parameters for the beating curves at these two time intervals or patterns may be compared through the comparison of the beating curves. When comparing curves it may be desirable to align curves to match an initial positive peak or initial negative peak. Aligning curves may also use a variety of curve algorithms, which identify distances or shifts between curves.

In one embodiment, the beating pattern similarity is derived as a parameter to compare the determined parameters for the beating curves at two time intervals. For example, one may compare the beating rates BR_1 and BR_2 at the two time intervals. An example of the beating pattern similarity is given as:

$$\text{Beating pattern similarity} = (2 * BR_1 * BR_2) / (BR_1 * BR_1 + BR_2 * BR_2)$$

With this above example, the beating pattern similarity is one (the highest value) when the beating rates at the two time intervals are the same. When the beating rates differ at two time intervals, the beating pattern similarity would be less than 1. The more the beating rates differ, the smaller the beating pattern similarity value.

In a preferred embodiment, however, the beating pattern similarity is derived as a parameter to directly compare the beating curves at the two time intervals. The idea of the beating pattern similarity should possess such properties that the value for beating pattern similarity is large when the two beating curves are similar, and the value for beating pattern similarity is small when the two beating curves are not similar. There are multiple methods for deriving such beating pattern similarity values.

In one method, as briefly mentioned above, for comparing the beating curves at two time intervals (assuming the same measurement time resolutions for the measured data points), it may be desirable to align curves to match an initial positive peak or initial negative peak. After aligning the initial peaks, an “AND” operation is performed on the time points for the two beating curves so that the overlapping time points on the two beating curves are kept whilst non-overlapping time points on either one of the beating curves are discarded. Thus, the remaining, overlapping data points on the two beating curves are of the same number and it is possible to readily define a distance to describe whether the two beating curves are similar. For example, the beating pattern similarity could be the correlation coefficient between the two data series in the remaining portions of the two beating curves. Clearly, the more similar the two curves, the larger the correlation coefficient (i.e., the larger the beating pattern similarity value is). In another example, the beating pattern similarity could simply be a certain mathematically-defined-distance (e.g. Euclidean distance) between two data series in the remaining portion of the two beating curves. Note that if the measurement time resolutions differ between the measured data points, additional time points may be artificially inserted into the beating curves with missing time points after mathematical interpolation of the values for such added time points based on other measured data points. With this operation, the two beating curves would have the same time resolutions.

In another method for comparing the beating curves at two time intervals (again, assuming the same measurement time resolutions for the measured data points), one would take the beating curve (out of the two) with the shorter time duration. If the time-shorter beating curve comprises more than half of the data points of the other beating curve, then some last data points from the shorter beating curve are removed to form a “base-curve” so that the number of the remaining data points in the shorter beating curve is half of the number of the data points in the other beating curve. Then a number of correlation coefficients would be determined where each correlation coefficient corresponds to the base-curve aligned to one continuous segment (comprising of the same data point number as the base-curve) of the other beating curve. For example, the first correlation coefficient is determined between the data series of the base curve and the data series of first half of the other beating curve (starting from the first data point). The second correlation coefficient is determined between the data series of the base curve and the data series from the

other beating curve with starting point being the second data point. The last correlation coefficient is determined between the data series of the base curve and the data series from the second half of the other beating curve ending with the last data point. Finally, the beating pattern similarity is determined as the maximum value of all the correlation coefficients.

There may be other methods or algorithms that could be used for deriving beating pattern similarities. Beating pattern similarity could be used to analyze the effect of a compound on the beating pattern of cardiomyocytes. The beating curves from two time intervals are compared. For analyzing the effect of a compound, one time interval corresponds to the time period before compound treatment whilst the other time interval corresponds to the time period after compound treatment. The beating pattern similarity has an advantage over other parameters in comparing compound's effect on the cardiomyocytes. The advantage is that it could include or summarize all the effects due to the compound, i.e. the effects on the beating rates, beating waveform shapes or beating amplitudes etc could all be included into the single parameter of the beating pattern similarity.

Beating rhythm irregularity (BRI) is a parameter which identifies changes in beating rate or changes between peak periods for a beating curve over a time interval. Beating rhythm irregularity is also referred to as a beating rate irregularity index. If the beating rate or the beating peak period does not change with time, then the beating rhythm is regular and the parameter of the beating-rhythm-irregularity should be small, i.e. being zero or close to zero. On the other hand, if the beating rate or beating period does change with time, then beating rhythm is irregular and the parameter of the beating rhythm irregularity should have a large value. As one requirement, the parameter of the beating rhythm irregularity should be able to identify the arrhythmic beating of cardiomyocytes. Thus, the beating-rhythm-irregularity should attain a large value for the beating curves of cardiomyocytes if the cardiomyocytes exhibit arrhythmic beating. There are multiple methods for calculating the beating rhythm irregularity for a beating curve over a time interval. For example, the positive peak periods for each-adjacent-positive-peak-pair are calculated for the beating curve in the given time interval. Then the average and standard deviation of such multiple positive peak periods are calculated. The beating rhythm irregularity can be calculated by dividing the standard deviation of the positive peak periods by the average. In another example, the negative peak periods for each adjacent negative

peak pair are calculated for the beating curve in the given time interval. Then the average and standard deviation of such multiple negative peak periods are calculated. The beating rhythm irregularity can be calculated by dividing the standard deviation of the negative peak periods by the average.

5 When the cardiomyocytes exhibit irregular beating, the beating curves may comprise some regular beating peaks (positive or negative) with regular amplitudes and some irregular beating peaks with somewhat smaller (/or larger) amplitudes or with somewhat different beating waveforms (e.g., the impedance value or cell index value or other measurement value does not return to baseline level after a beating
10 peak) . Thus, number or presence of the irregular positive (or negative) beating peaks could be used as an indicator for beating rhythm irregularity. Such regular and irregular beats can be determined and the ratio between the irregular and the regular beats, i.e. irregular to regular beats ratio, can be used to assess the pro-arrhythmia effects of the test compound. If there are no pro-arrhythmia effects, such ratio should
15 be very small and close to zero.

 The skilled artisan will appreciate compounds may be added to cardiomyocyte populations to test for potential changes or effects in beating parameters. Changes may include an increase or decrease in at least one of the beating parameters. The change may include an increase in beating rhythm irregularity if the compound causes
20 an irregular beating in cardiomyocytes. If a compound results in a significant effect on the beating of cardiomyocytes, then beating pattern similarity between the beating curves before and after the compound treatment may be small.

 In some instances beating measurements are continually monitored and a beating parameter is calculated for a single beating cycle within a time period for
25 comparison. For example, the calculation for a beating parameter such as an amplitude can be performed for a single beating cycle. Further, a beating parameter from a single beating cycle (i.e. single beating peak) may provide a suitable control for later determined beating parameters. Due to the fact that single beating cycle may take a short time to complete, beating parameter for a single beating cycle (i.e. single
30 beating peak) is sometimes referred as beating parameter at a single time point.

 Frequently, it may be desirable analyze one or more parameters within a single beating period (i.e. for a single beating cycle). From a single beating cycle (single beating period) a variety of beating parameters can be assessed including beating amplitude, rising or falling time, rising and falling slope and IBD50 and the like.

Consideration within a beating period may also be particularly useful for use as an initial beating period before the addition of a test compound to establish control beating data for later comparison. The single beating period approach may also be of interest if an irregularity is detected, such as an irregular positive peak, negative peak, slope between peaks and the like. For instance, an irregularity may be temporal and thus decay over time, which if limited to studying across multiple beating periods could be averaged out and thus not be identified as statistically significant. Thus, if such an irregularity is identified, analysis of the corresponding beating period may reveal significant differences compared to control. Still further, analysis of the single beating period may reveal a correlation to another change or shift in beating parameter not apparent through multi-beating period analysis.

In another approach one or more of the selected parameters are determined or calculated across a time period that includes multiple beating periods (i.e. multiple beating cycles). One or more parameters can be determined for each of the desired multiple beating periods. This approach may be used to monitor consistency over time or as a continuous scanning technique to identify potential periods of irregularity for further single beating period analysis. The approach may also be used to develop average values for comparison to test compounds.

While the methods permit the study of cardiomyocyte beating itself including, changes in beating in response to cardiomyocyte or stem cell differentiation and the like, the methods are also useful to assess the effect of one or more test compounds on a cardiomyocyte cell population. Such assays may help predict potential cardiotoxic effects of compounds, effect on cell differentiation or growth and the like. The skilled artisan will appreciate parameters for cell populations treated with test compounds may be compared to control treated populations, such as vehicle control, and the resulting parameters compared to assess for differences. Differences in beating parameters may be indicative of cardiotoxicity or other effects.

In another embodiment, a dose response curve (DRC) of a test compound is determined. A dose response curve reveals changes to beating parameters in response to difference in concentrations of test compound. Preferably, selected parameters are calculated from beating measurements that are measured from wells having different concentrations of the same test compound for a period of time where beating signals are continuous. Preferably, for each concentration, one value is calculated for the selected parameter for certain time points after addition of test compound. Then a

concentration dependent dose response curve of the selected parameter is fitted to a non-linear, sigmoidal-dose-response equation to derive EC50 (or IC50) values. An example of a sigmoidal dose-response curve is as follows:

5
$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{Log EC}_{50} - X)})$$

An example of a sigmoidal dose-response with variable slop is as follows:

10
$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{Log EC}_{50} - X) * \text{HillSlope}))}$$

The derived IC50 / EC50 values for different compounds could be used to predict, analyze, or compare the different compounds' cardiotoxicity or different compounds' effect on beating of cardiomyocytes. Like IC50 or EC50 derived from other assays, a compound with lower IC50/EC50 values may indicate that the
15 compound is more potent in inducing cardiotoxicity effect or causing an effect on beating of the cardiomyocytes than the compound with higher IC50/EC50 values.

To further demonstrate the use of the above parameters, modulators of ion channel and non-ion channel proteins and targets as well as modulators of neuro-hormonal machinery were tested in both time and dose-dependent manner (see for
20 example **FIGS. 3A-C** and **FIGS. 4A-C**). Using various parameters such as beating rate, amplitude and IBD50, we have confirmed that the half maximal concentrations obtained for the different compounds are within the sensitivity range of patch clamp recording and other electrophysiological methodologies (data not shown). Importantly, we have also shown that certain classes of compounds such as
25 blebbistatin, which serve to decouple excitation from contraction, can be missed by electrophysiological readouts but can be detected by the impedance-based measurement of cardiomyocyte beating. These observations demonstrate that impedance-based assay system can detect modulation of ion-channel and non-ion channel targets affecting both excitation and contraction of cardiomyocytes.

30 Also demonstrated is that a number of drugs that have been shown to induce ventricular arrhythmia in the clinic and subsequently withdrawn from the market display reproducible impedance-based signature beating profiles in a time and dose-dependent manner (**FIGS. 13A-B** and **FIG. 15**). These signature beating profiles are qualitatively similar to early after depolarization (EAD) phenomenon that is observed

by manual patch clamp recording of cardiomyocytes after exposure to compounds and drugs that inhibit ERG channel activity and consequently I_{kr} current (Fermini *et al.*, 2003). While the appearance and mechanism of EADs are diverse, it is generally agreed that calcium homeostasis which can influence contraction plays an important
5 role in various forms of EADs (Volders *et al.*, 2000). It is thought that drug-induced block in I_{kr} current can delay repolarization leading to delayed inactivation of calcium channels and consequently, the resulting late inflow of calcium contributes to emergence of EADs (Fermini *et al.*, 2003). It is also important to mention that manifestation of arrhythmia by mechanisms other than ERG block and QT
10 prolongation cannot be discounted. For example, it has been shown that certain compounds which shorten both APD and QT interval can also cause arrhythmia (Lu *et al.*, 2008). While it remains to be shown whether such drugs can also induce characteristic EAD-like profiles in the assay system described here, it underscores the need for implementation of an assay system which can assess the integrated response
15 of ion channel and non-ion channel targets to capture the full range of drug responses.

While the cardiotoxic side effect of most drugs is primarily viewed as a function of the concentration of the drug and its possible adverse interaction with other compounds, it must be emphasized that the time-dependent response of cardiomyocyte to a given concentration exposure is also an important parameter. This
20 point is especially relevant for cardiac function where time-dependent heart rate variability or dynamics of periodicity could be a key determinant of proarrhythmic potential (Bass *et al.*, 2008). Furthermore, an increasing number of drugs are found to inhibit hERG function by dual mechanisms of short-term channel block and long term hERG trafficking defects that operate over different time and concentration scales
25 (van der Heyden *et al.*, 2008). The non-invasive nature of the impedance readout allows for monitoring of cardiomyocyte response from millisecond time frame to minutes, hours and days in real-time and therefore well suited to capture time series data for these complex responses. The utility of this feature was demonstrated with respect to treatment of mouse embryonic stem cell derived cardiomyocytes
30 (mESCC) with both pentmidine and doxorubicin (**FIGS. 14A-C**). Pentmidine, a hERG trafficking inhibitor associated with TdP in the clinic had a major impact on beating rate and beat duration that was elicited about 900 min after initial exposure (Kuryshhev *et al.*, 2005). Standard manual patch clamp techniques or planar patch clamp techniques, while the golden standard, and most likely would have missed this

effect because action potential recordings are typically performed within the first hour after compound exposure. Doxorubicin, an anthracycline affected both the beating rate and periodicity of beating while causing progressive and dose-dependent decrease in overall impedance signal of the cells (within 24 hours after compound exposure) most likely due to loss of viability (**FIGS. 14A and B**). Therefore, the ability to simultaneously capture the dynamicity of beating rate changes coupled with overall changes in impedance to reflect the global compound effect on cell health within the same assay can provide additional information regarding mechanism of toxicity.

10 EXAMPLES

Example 1: Detection of changes in cardiomyocyte beating in response to administration of pharmacologically active agents

15 Cell culture. Mouse ES cell-derived cardiomyocytes (Cor.At) were obtained from Axiogenesis (Cologne, Germany, catalogue number XCAC-1010E, Lonza Cologne). The cells were kept in liquid nitrogen until thawed and cultured according to protocol provided by Axiogenesis with slight modifications. Briefly, each well of the E-PLATE (ACEA Biosciences Inc., San Diego, CA) was coated with 50 μ l of a 1:100
20 diluted fibronectin (FN) solution (F1114, Sigma-Aldrich, USA) and incubated at 4°C over night. Subsequent to removal of FN, the wells were washed with PBS and followed by cell seeding. The cells were thawed at 37°C in a waterbath, transferred to 15 mL conical tube containing 9 ml fresh Cor.At complete culture medium (XCAM-250E, Lonza Cologne, Germany), centrifuged at 100g for 5 minutes and the medium
25 was replaced with small volume of fresh Cor.At complete culture medium containing puromycin at final concentration of 10 μ g/ml. The cells were counted and the percentage of viable cells determined by trypan blue exclusion method.

Monitoring of cardiomyocyte attachment and contraction. About 40000 - 60000
30 viable cells were seeded per well of a 96 well E-PLATE (ACEA Biosciences Inc., San Diego, CA) and the cells were monitored using the xCELLigence RTCA Cardio system (Roche Applied Science and ACEA Biosciences). Cell culture medium was replaced once on a daily basis. Typically, drug treatment was initiated 60-80 hours after cell seeding depending on seeding density. Data collection is controlled by

software program which operates the hardware and allows the user to define the sampling frequency and sampling window. Sampling frequency is defined as the number of times during an experimental run the beating is sampled and the sampling window is defined as the duration of time that the beating is actually measured. For example if the sampling frequency is 15 minutes and sampling window is for 5 second means that each 15 minute the system will record beating data for 5 seconds. In a typical experiment, prior to compound treatment the sampling frequency is once every hour and the sampling window is 20 seconds. 5 min prior to treatment, the cells are sampled every minute for 20 seconds to establish baseline recording. After treatment, the sampling frequency is every minute for the first hour, every 5 minutes for the second hour and every 15 minutes for 3-24 hours. The sampling window for each recording is fixed at 20 seconds. After the data acquisition, the RTCA Cardio software is used to calculate the parameters such as beating rate, amplitude, beating period, normalized beating rate, normalized amplitude, and beating rate irregularity (BRI) index and perform subsequent basic statistics, like average and standard deviation and further supply EC50 values for dose-response testing.

Terms and Analysis Parameters. Each measured beating cycle corresponds to the excitation-contraction coupling of the cardiomyocytes. The typical measured beating pattern is illustrated in **FIG. 7** and **FIG. 8**. The beatings are composed of a sequence of positive peaks (+P) and negative peaks (-P). The Cell Index difference between one negative peak to the following positive peak is defined as amplitude. The time between each positive peak is defined as beating period and the beating rate is calculated based on each beating period to derive how many beatings occurred in one minute. Three time-related parameters, rise time T_r , falling time (T_f or termed as decay time T_d), and half-amplitude width T_{IBD50} , resolve the temporal beating characteristic.

For data analysis, the related parameters are calculated for every beating within one recording period and the average and standard deviation are derived correspondingly. In order to compare the effect of tested compounds, beating rate or amplitude after compound treatment are normalized to the same time point before compound treatment to obtain the normalized beating rate or normalized amplitude. In order to evaluate the degree of arrhythmia, the beating rhythm irregularity (BRI) index is

derived based on the coefficient of variation (i.e. standard deviation divided by average) of the beating period during one record period.

Multi-Electrode Array. For the culture of the mouse ES cell-derived cardiomyocytes
5 a sterilized substrate-integrated planar standard MEA (59 TiN electrodes and a
grounded reference electrode, 8x8 electrode grid, electrode spacing 200, electrode
diameter 30, glass ring (Multi Channel Systems GmbH, Reutlingen, Germany), 10 μ l
of the 1:100 diluted fibronectin solution (F1141, Sigma-Aldrich) was placed exactly
on the microelectrode area of the MEA and incubated for at least 3 hours at 37°C in
10 an humidified incubator. Afterwards residual coating solution of taken off and 20 μ l
with 2×10^4 cardiomyocytes were placed on the coated electrode area and the
complete MEA was incubated for another 3 hours at 37°C in the incubator to establish
cell adhesion before 1 ml of Cor.At culture medium was applied. The MEA was
connected to the amplifier and data-acquisition system (Multi Channel Systems) with
15 band pass filter characteristics of 0.5 Hz to 1 kHz. Spontaneous electrical activity was
recorded with software (MC Rack; Multi Channel Systems) (Stett 2003). Data were
recorded simultaneously from 59 channels with a sampling frequency of 10 kHz.

Cardiomyocytes on MEAs were kept in an incubator at 37°C during the whole time
20 period of the assay. The cells were equilibrated to the assay buffer (IMDM + 0.1%
FCS) for at least 45 min prior to baseline recording and subsequent substance
application. After that, three increasing concentrations of the test compound were
applied consecutively for 15 min each. Additional wash-out period with was at least
45 min. Analyzed parameters from extracellular recordings did not alter in a time
25 dependent manner in time-matched control experiments of the vehicle (water or 0.1%
DMSO) during all experimental phases.

Raw data from electrode array recordings was analyzed offline. Frequency was
determined as the reciprocal value of the inter spike intervals of the field action
30 potentials and field action potential duration was calculated according to Halbach et
al. (2003) (Halbach *et al.*, 2003). Frequency correction of the field potential duration
was assessed according to Mitchell et al. (Mitchell *et al.*, 1998).

Data are presented as mean values $\pm$ standard error of the mean in percent of
35 baseline. In order to evaluate compound-induced effects relative to control

measurements, differences between the control group and the compound measurements were tested for statistical significance by means of unpaired Student's t-test.

5 Microelectronic monitoring of cardiomyocyte beating. To characterize the beating, mESCC were seeded in the wells of the E-Plate at a density of 40,000 cells/ well. The cells were monitored up to 96 hours in culture (**FIG. 9A**) and the beating activity was recorded at 12, 24, 48, 72 and 96 hours (**FIG. 9A**, arrows) for a total duration of 20 seconds (**FIG. 9B**). Interestingly, within 24 hours after seeding the cells no consistent
10 beating activity could be detected even though, under the microscope clusters of asynchronously beating cardiomyocytes, which have not had an opportunity to form a syncytium could be detected (data not shown). However, within 48 hours the individual clusters begin to form clear connections and the entire monolayer of cardiac cells in the bottom of the well begin to beat in a synchronous manner.
15 Likewise, based on impedance recording, reproducible beating activity is detected by 48 hours (**FIG. 9B**). The beating rate at 48 hours is approximately 80 beats/minute and progressively increases with time reaching almost 250 beats/minute after a month in culture. These observations are consistent with electrophysiological monitoring of action potential duration in mouse ES cell-derived cardiomyocytes (Fijnvandraat *et*
20 *al.*, 2003).

In order to analyze the curves and quantify beating activity, 3 different analysis parameters were derived; T_{IBD50} , T_r and T_d . T_{IBD50} is a parameter which measures the duration (ms) between the rise and fall of beat cycle at 50% of maximal amplitude.
25 T_{IBD50} values for mESCC at corresponding times are shown in **FIG. 9B**. At 48 hours the T_{IBD50} value is 142 ± 4.6 ms which decreases to 105 ± 2.4 ms by 96 hours. The initial rise in amplitude denoted as T_r is relatively fast and depending on the time of recording can vary from 29 ± 5.1 ms to 38 ± 1.4 ms (**FIG. 9B**). The decay time (or the falling time as shown in **FIG. 8**), denoted as T_d , which reflects the time the signal
30 decays from 80% of peak height to 20% of peak height is longer compared to T_r and can range from 88 ± 7.2 ms to 124 ± 12.0 ms, depending on the time of recording (**FIG. 9B**). Interestingly, the kinetics of rise and fall of impedance mirrors that of calcium in mouse embryonic cardiomyocytes (Rapila *et al.*, 2008) and it is possible

that T_r and T_d may reflect the time for two alternating phases of the beating cycle, namely contraction and relaxation.

To determine if the impedance signal is reflective of physical contraction and relaxation cycle of mESCC, we used an inhibitor of the myosin heavy chain ATPase activity, blebbistatin, shown to inhibit cardiomyocyte contraction (Kovacs *et al.*, 2004). As shown in **FIG. 9C**, blebbistatin treatment of mESCC resulted in significant inhibition of impedance signal, which was restored after washing the wells and culturing the cells in media without blebbistatin. Interestingly, at concentration which blebbistatin inhibited impedance measurement of beating activity, no effect on action potential duration was detected using field potential recording (**FIG. 9D**). The inability of electrophysiological readout to detect blebbistatin effect has also been confirmed using isolated rat and mouse ventricular myocytes, isolated rabbit ventricle, and Langendorff-perfused rabbit hearts (Dou *et al.*, 2007; Fedorov *et al.*, 2007) indicating that electrophysiological readouts may miss the potential side effect of compounds depending on whether the compounds affect the electrical or mechanical aspects of cardiomyocyte contraction. Overall, the results presented thus far demonstrate that impedance readout can be used to monitor the rhythmic contraction/relaxation cycle of mESCC in culture over a prolonged duration and in combination with electrophysiological readouts may be able to detect compounds that decouple excitation and contraction.

Pharmacological assessment of mESCC using impedance monitoring. Using specific pharmacological modulators of ion channel and non-ion channel targets, we set out to dissect specific events of the excitation/contraction cycle in mESCC. First, the time and dose-dependent effect of various ion channel modulators of calcium, sodium and potassium channels were tested (**FIGS. 10A-C** and **FIGS. 11A-C**). For these experiments mESCC were thawed, seeded in the wells of the E-Plate, cultured for 3 days, treated with increasing concentrations of the compounds and monitored for 24 hours using the RTCA Cardio system.

Assessment of voltage-gated calcium channels. Embryonic stem cell-derived cardiomyocytes are known to undergo spontaneous contractions due to intracellular

calcium oscillations mainly initiated from the sarcoplasmic reticulum (SR) (Sachinidis *et al.*, 2003). It is also believed that during SR-driven spontaneous activity, the plasmalemmal voltage activated calcium influx could provide a compensatory mechanism for restoring depleted calcium pools in the SR (Rapila *et al.*, 2008). Application of isradipine, a well known voltage activated L-type calcium channel blocker of the dihydropyridine class (Triggle, 2003) caused a progressive time and dose-dependent decrease and inhibition of beating activity, indicating that calcium entry through L-type calcium channels is required for beating (**FIG. 10A**). The half maximal dose-response value for isradipine induced inhibition of beating activity based on measurement of normalized beating rate and amplitude is 19.7 nM and 42.3 nM respectively (at 5 min time point after compound addition; **FIG. 12**). These values are consistent with efficacy of isradipine tested in isolated rabbit heart (Mellemejaer *et al.*, 1992) as well as recombinant HEK-293 cells stably expressing the human Cav1.2 (Balasubramanian *et al.*, 2009) (**FIG. 15**). The compound (S)-(-)-Bay K 8644 is also of the dihydropyridine class, but acts in an agonistic mode to activate voltage-gated calcium channels (Franckowiak *et al.*, 1985; Schramm *et al.*, 1985). Treatment of mESCC with (S)-(-)-Bay K 8644 resulted in a dose and time dependent effect that substantially increased the beating rate which persisted for up to 12 hours at higher concentration and declined by 24 hours (**FIG. 10B**). The increase in beating rate is consistent with the reported ionotropic action of this compound and the EC50s obtained (77 nM) for beating rate is consistent with previously published reports using rat ventricular myocytes (Zahradnikova *et al.*, 2007) (33 nM; data not shown).

Assessment of potassium channel modulators. Next, the effect of Chromanol 293B, an inhibitor of slow activating delayed rectifier K⁺ current (I_{ks}) (Bosch *et al.*, 1998; Fujisawa *et al.*, 2000; Ono *et al.*, 2000) was tested (**FIG. 10C**). While at the highest dose (100 μM) Chromanol 293B treatment resulted in complete inhibition of cardiomyocyte beating activity, at intermediate doses it slows down the beating rate (69% and 80% of control at 25 μM and 3.13 μM, respectively 5 min post compound addition) and also prolongs the beat duration (13.0 ms and 21.1 ms at 25 μM and 3 μM respectively and at 5 min post compound addition). The I_{ks} is mainly involved in the repolarization phase of the action potential and its inhibition by Chromanol 293B

leads to increased action potential duration (APD) of canine ventricle myocytes(Volders *et al.*, 2003) and stem cell-derived human cardiomyocytes (Peng *et al.*) as measured by electrophysiological techniques. The increased APD has been shown to slow down the decline of calcium concentrations and thereby may prolong the contraction phase of cardiomyocytes (Bouchard *et al.*, 1995).

The rapid activating component of the delayed rectifier current (I_{Kr}) is also involved in the repolarization phase of cardiac action potential and is mainly mediated through the ERG channel (Brown, 2005). The effect of E4031, a potent ERG channel inhibitor, was also tested using mESSC in a time and dose-dependent manner (**FIG. 11A**). As shown, E4031 treatment interrupted that normal rhythmicity of beating, especially at high concentrations (200 nM-800 nM) and resulted in prolonged beat durations which are accompanied by plateau oscillations. This phenomenon was typical of other ERG blockers as well (see next section). At the doses tested the cells appear to recover from the effect of E4031 by 24 hours after treatment. Based on normalized beating rate and beat rate irregularity parameter, the half maximal value obtained is 27 nM and 57 nM, respectively and is consistent with the reported IC_{50} for E4031(10 nM) using stem cell-derived human cardiomyocytes with patch clamp technique (Peng *et al.*).

Assessment of sodium channel modulators. Voltage-gated Na^+ channels are primarily responsible for the Na^+ current and the depolarization phase of cardiac action potential. Based on gene expression and electrophysiological data, the *Scn5a* gene product, which encodes for the α -subunit of voltage-gated Na^+ channel, is present and functional within mESCC. Treatment of mESCC with Tetrodotoxin (TTX), a potent and selective inhibitor of voltage-gated Na^+ channels (Narahashi, 2008), led to a dose-dependent decrease in beating rate of mESCC which is sustained at the higher concentrations for the entire duration of 24 hours (**FIG. 11B**). The apparent half-maximal dose-response value obtained for inhibition of mESCC beating is about 0.28 μ M (**FIG. 12**).

Assessment of chronotropic agents. Activation of the sympathetic nervous system and neuro-hormonal regulation through the β -adrenergic receptor is a major

mechanism controlling rate and contractility of the cardiac tissue (Bers, 2002). The protein machinery responding to β -adrenergic receptor stimulation is present and functional within mESCC and its agonists are well characterized chronotropic and ionotropic stimulants (Maltsev *et al.*, 1999). Therefore we sought to test whether β -adrenergic receptor stimulation could be detected by the RTCA Cardio system. Treatment of mESCC with Isoproterenol, a β -adrenergic receptor agonist, increased the contraction frequency of mESCC in a dose and time-dependent manner while decreasing the overall duration of each beat (**FIG. 11C**). The overall effect is similar to the L-type calcium channel agonist (S)-(-)Bay K 8644 (**FIG. 10B**) and is consistent with the observation that stimulation of β -adrenergic receptors leads to activation of L-Type calcium channels (Maltsev *et al.*, 1999). The data presented in this section demonstrates that impedance is a sensitive readout to assess cardiomyocyte beating and its modulation by pharmacologically active compounds which alter cardiac electrophysiological and/or contractile properties. Compound treatment produces both time and concentration-dependent responses which are captured in real-time by the RTCA Cardio system. Both acute and long term assessment of beating activity may provide additional mechanistic insight as demonstrated for ERG channel inhibitors in the next section.

Example 2: Mechanism-dependent compound toxicity screening: Identification of impedance-based pro-arrhythmic signatures

To test the utility of RTCA Cardio system for pre-clinical cardio-safety screening two complementary approaches were undertaken. First, 4 drugs withdrawn from the market due to increased incidence of TdP (Fermini *et al.*, 2003) were screened in a dose-response manner using mESCC (**FIG. 13A**). These compounds have subsequently been shown to also inhibit hERG channel activity (Brown, 2005). All four compounds significantly affected beating rate in a dose-dependent manner (**FIG. 13A**) and produced beating irregularities that were consistent with those observed for E4031 in terms of beating waveform, suggesting a common underlying mechanism (**FIG. 13B**). We speculate that the plateau oscillation phenomenon observed by hERG channel blockers may be related to early after depolarization (EAD) effect caused by blocking of the hERG current leading to premature activation of voltage-dependent L-

Type calcium channels, calcium entry and culminating in premature contractions (Rubart *et al.*, 2005). These signature beating waveforms were also observed for other drugs that are known to interact with and block ERG activity (**FIG. 15** and see below). In order to better quantify the beating irregularities we derived a kinetic parameter referred to as beating rhythm irregularity (BRI) index which represents the coefficient of variation (standard deviation divided by average) of beating rate periods. Based on this parameter we derived half maximal concentrations for E4031 (**FIG. 12**), astemizole, cisapride, droperide and sertindole (**FIG. 13A**) which are 57 nM, 290 nM, 2700 nM, 2100 nM and 290 nM, respectively. The respective values obtained here are within the range reported for these compounds using electrophysiological methods (data not shown). These findings suggest that impedance-based beating profiles could be used in a predictive manner to screen for and identify compounds that may have off-target interactions with ERG channel.

Next, a compound library containing 50 proarrhythmic and antiarrhythmic compounds was also screened at 3 doses (10 μ M, 1 μ M and 0.1 μ M) (**FIG. 15**). As shown, all known hERG blockers with the exception of Terfenadine displayed beating profiles consistent with those shown in **FIG. 13B**. A more extensive dose-response profiling of Terfenadine may be required in order to observe the signature beating profile similar to other hERG channel inhibitors. In addition, in this screen, compounds modulating other ion channel targets such as calcium and sodium also profoundly and dose-dependently affected beating activity.

Example 3: Assessment of short and long term cardiac liability using impedance-based systems

The true test of any *in vitro* assay utilized in preclinical safety assessment depends on its ability to model and predict *in vivo* effect in the clinic. Thus far we have shown compounds modulating ion channel activities in cardiomyocytes can be detected by the RTCA Cardio system. However, there are a number of drugs whose cardiac liability in the clinic extends beyond its propensity to just cause arrhythmia; for example the chemotherapeutic agent, doxorubicin, has been shown to induce arrhythmia (Singal *et al.*, 1998) as well as cardiotoxicity by interfering with mitochondrial function (Minotti *et al.*, 2004). Therefore, we wanted to determine if

the RTCA Cardio system in combination with mESCC can model and predict the complex effects of doxorubicin. As shown in **FIG. 14A**, treatment of mESCC with doxorubicin results in time and dose-dependent decrease in global impedance readout, presumably due to loss of cardiomyocyte viability. Likewise, **FIG. 14B**, shows the
5 dose- and time-dependent effect of doxorubicin on cardiomyocyte beating within the same assay. Doxorubicin treatment of mESCC leads to significant decrease in overall beating rate and also induces irregular beating pattern that has features of compounds that induce arrhythmia. While it may be difficult to draw direct parallels between in vitro assays and clinical observations, both acute and chronic effect of doxorubicin
10 resulting in arrhythmia has been documented in the clinic (Minotti *et al.*, 2004).

The mode of interaction of drugs with various targets within cardiomyocytes may be direct as shown for the various ERG channel blockers and those of sodium and calcium channels or it could also be indirect affecting such processes as the folding or
15 transport of ion channel proteins to the membrane surface of cardiomyocytes (Dennis *et al.*, 2007) and therefore may go undetected in most conventional safety studies which are geared towards identification of direct ERG blockers. This point is best exemplified by the compound pentamidine, which in the U.S. is used as a second line of treatment of *Pneumocystis carinii* pneumonia, a common opportunistic infection in
20 patients with impaired immune function. Pentamidine has been shown to affect the transport of the ERG channel to the membrane in heterologous expression systems as well as in cardiac myocytes with repolarization being delayed as a direct consequence (Dennis *et al.*, 2007; Kuryshv *et al.*, 2005). Since this compound affects
25 ERG channel activity indirectly, its effect will be manifested in a time-dependent manner and difficult to capture by standard patch clamp techniques which are limited to the first hour of recording time. We tested the effect of pentamidine on mESCC in a time dependent manner (**FIG. 14C**). Administration of pentamidine at a final concentration of 20 μ M has no noticeable effect on beating rate and amplitude well
30 into 900 min after compound addition, at which point the beating rate slows down and the beating duration is significantly delayed, most likely due to extended repolarization phase. These observations highlight the importance of monitoring compound effect in a time-dependent manner in order to resolve the effect of

compounds on both early and longer term function of cardiomyocytes and to obtain greater mechanistic understanding.

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- 35

CLAIMS:

What is claimed is:

- 5 1. A method of determining a beating parameter for cardiomyocyte
beating analysis, comprising:
- 10 a) providing a cell analysis device comprising wells, each well
comprising a sensor capable of monitoring beating of cardiomyocytes in
millisecond time resolution;
- 15 b) adding cardiomyocytes to the wells;
- c) monitoring the beating of the cardiomyocytes in millisecond time
resolution to obtain a plurality of beating measurements and optionally
cell index; and
- 20 d) calculating one or more beating parameters from the plurality of
beating measurements or cell index.
2. The method according to claim 1, wherein the cell analysis device is an
impedance monitoring device and wherein each sensor comprises two electrode
structures, each having substantially the same surface area.
- 25 3. The method according to claim 1, wherein the sensor is an optical
sensor.
4. The method according to claim 3, wherein the optical sensor comprises
30 a resonant waveguide or a resonant waveguide grating sensor.
5. The method according to claim 1, wherein the step of calculating one
35 or more beating parameters comprises plotting the plurality of beating measurements

or optionally cell index over a time course to form a beating curve and deriving the one or more beating parameters from the beating curve.

6. The method according to claim 1, wherein the one or more beating parameters are selected from the group consisting of beating rate, beating amplitude, rising time, falling time, beating period, IBD10, IBD50, IBD90, rising slope and falling slope.

7. The method according to claim 1, wherein the one or more beating parameters are selected from the group consisting of normalized beating rate, normalized beating amplitude, beating pattern similarity and beating rhythm irregularity.

8. The method according to claim 1, wherein the one or more beating parameters comprises 5 beating parameters.

9. The method according to claim 1, wherein the one or more beating parameters are calculated while the device continues to monitor the beating of the cardiomyocytes.

10. A method of determining a dose response for a test compound on a cardiomyocyte population, the method comprising:

a) determining a beating parameter for different doses of a test compound according to the method of claim 1, wherein the test compound is added at different doses to the wells of the device; and

b) plotting the beating parameters for each dose to form a dose response curve.

11. The method according to claim 10, wherein the beating parameter for each dose is from a same time point.

12. The method according to claim 10, further comprising determining a IC50 or EC50 value from the dose response curve.

13. A method for identifying a compound having a potentially cardiotoxic effect, comprising:

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a) providing a test compound suspected of having a cardiotoxic effect;

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b) performing the method according to claim 1 to obtain a beating parameter for the test compound, wherein the test compound is added to the wells; and

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c) comparing the beating parameter for the test compound to a control beating parameter to identify whether there is a difference between beating parameters and if so, concluding the test compound has the potentially cardiotoxic effect.

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14. The method according to claim 13, wherein the beating parameter for the test compound comprises 2 beating parameters, which are compared to 2 control beating parameters, further wherein the test compound is concluded to have a cardiotoxic effect if at least one of the compared parameters is different.

25

15. The method according to claim 13, wherein the compound is suspected of being a pro-arrhythmic drug that may induce arrhythmia.