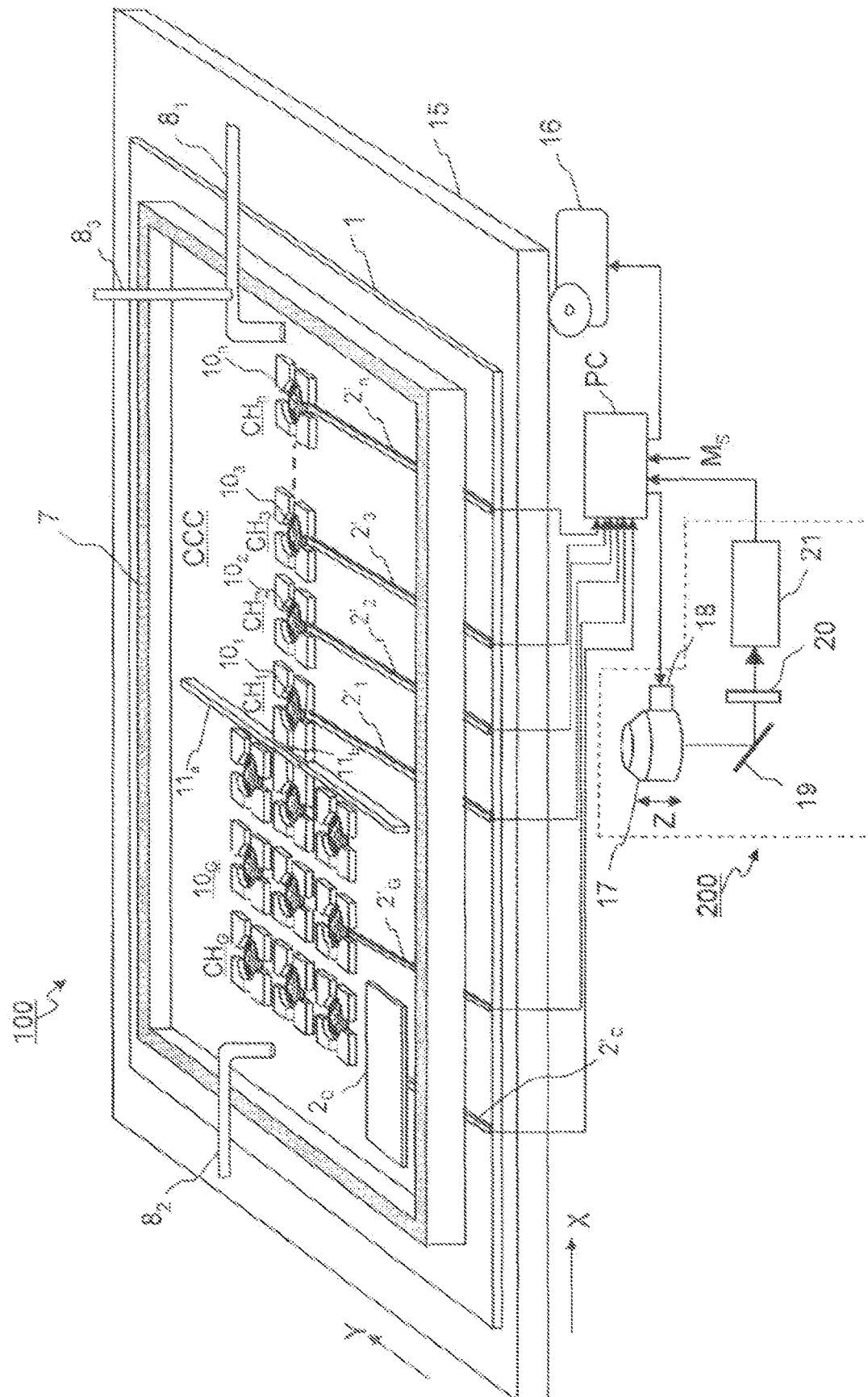
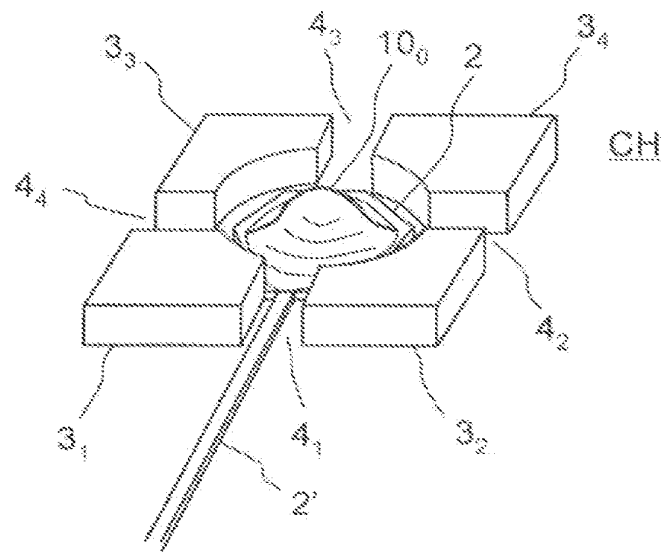


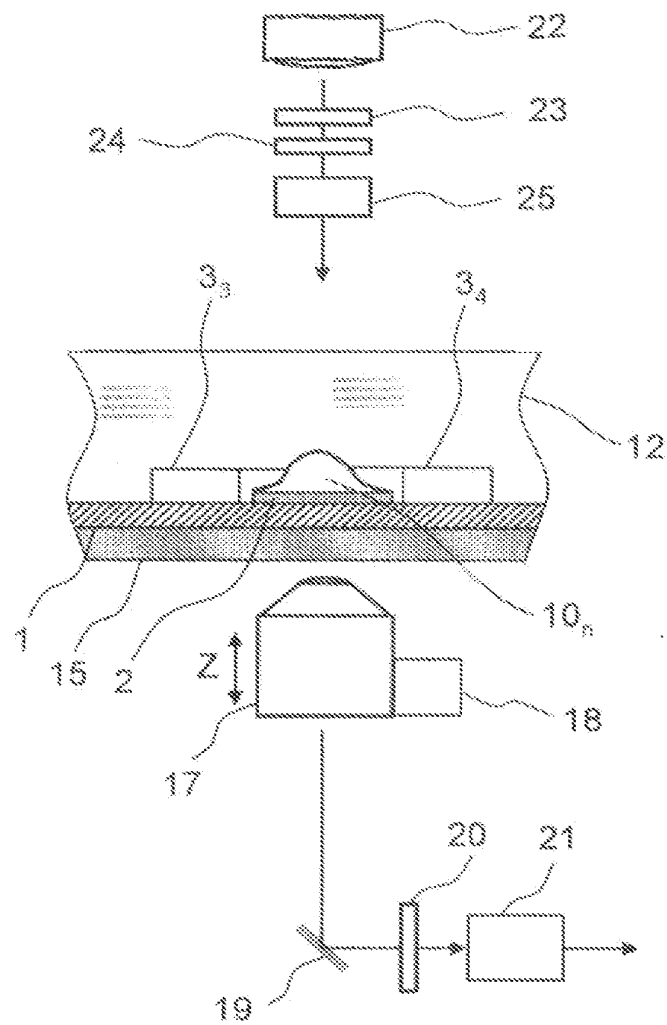
[図1]



[図2]

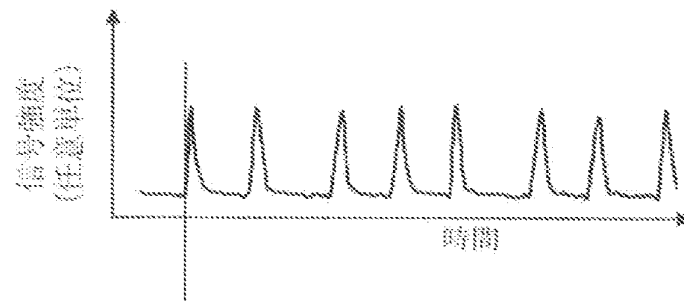


[図3]

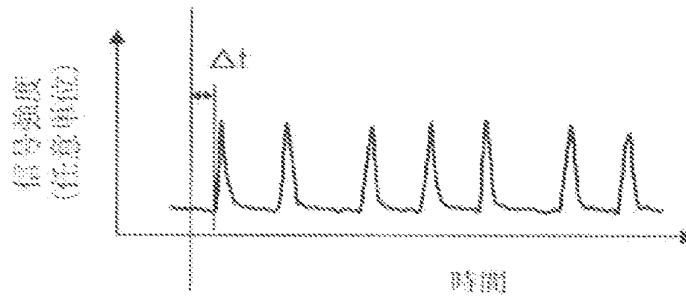


[図4]

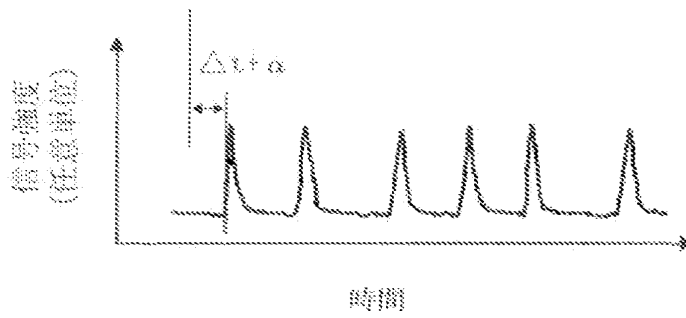
(a) 細胞集団の拍動



(b) 標的細胞の拍動 (通常時)

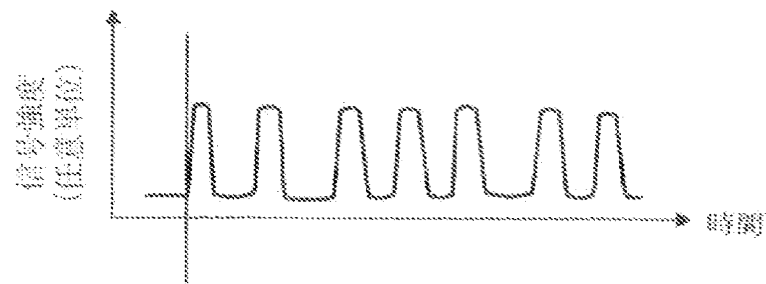


(c) 標的細胞の拍動 (薬作用時)

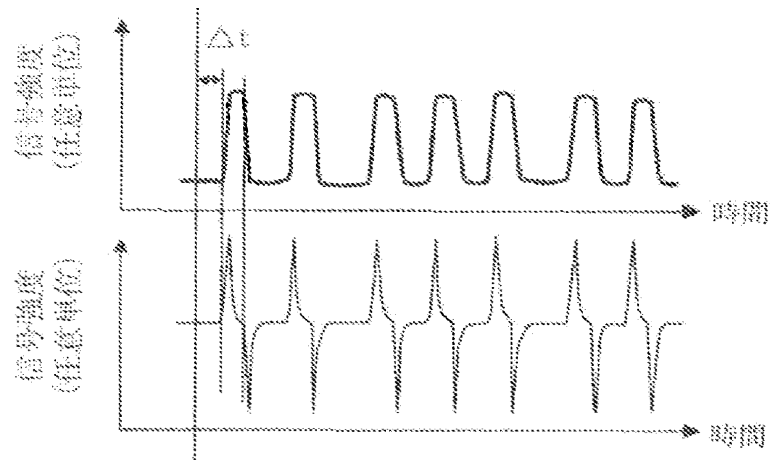


[図5]

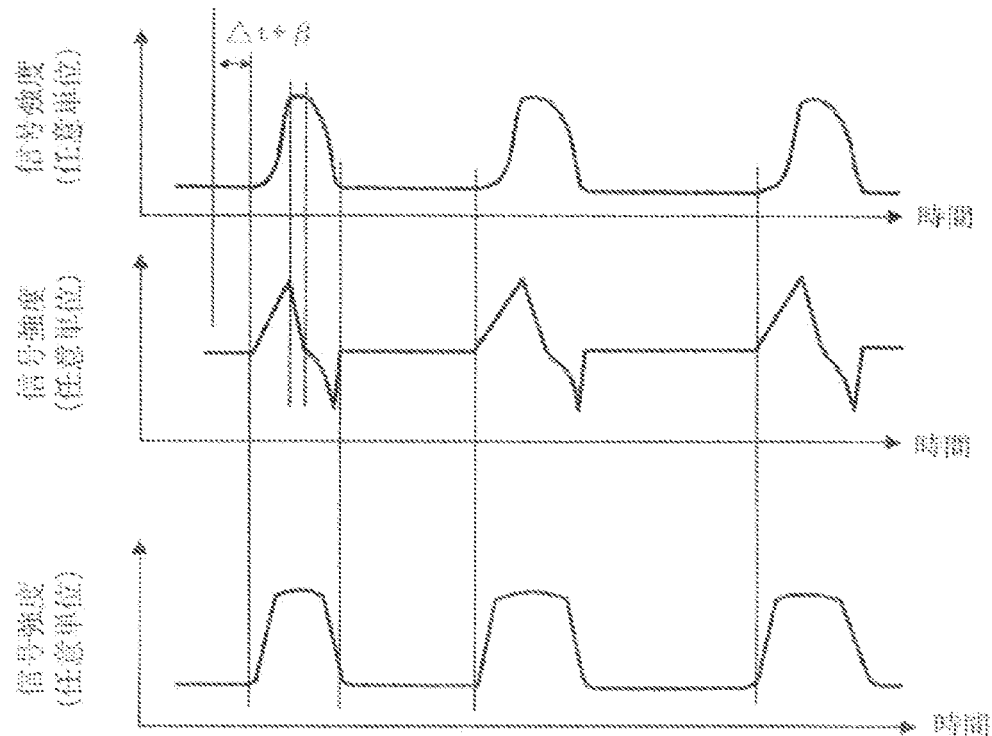
(a) 細胞集団の拍動による細胞の体積変化



(b) 標的細胞の拍動による細胞の体積変化 (通常時)

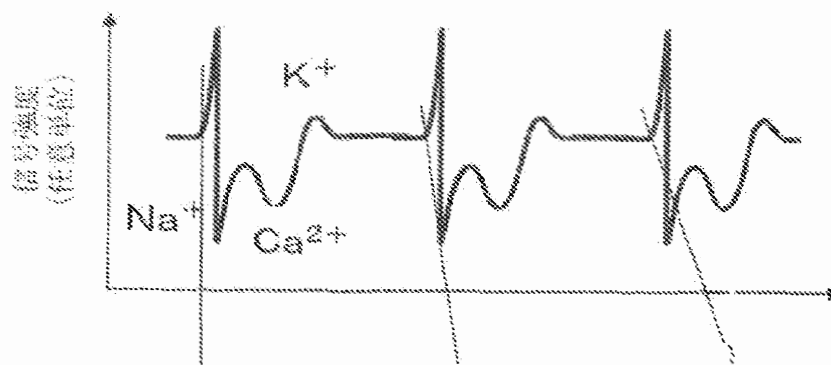


(c) 標的細胞の拍動による細胞の体積変化 (薬作用時)

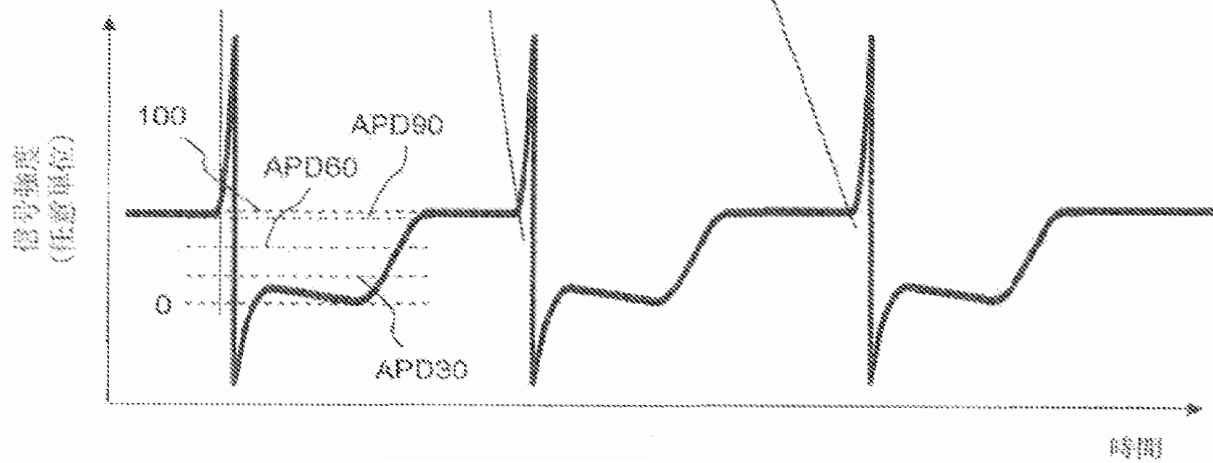


【図6】

(a) 標的細胞の細胞外電位変化 (通常時)

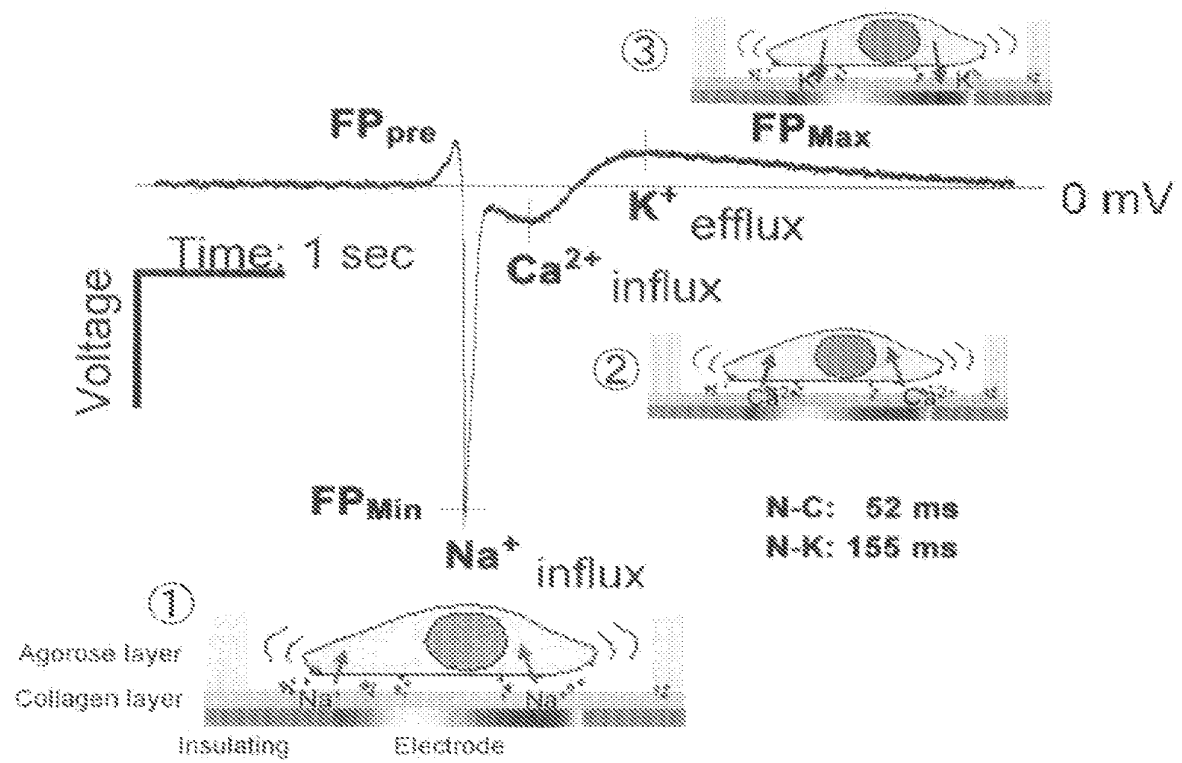


(b) 標的細胞の細胞外電位変化 (薬作用時)

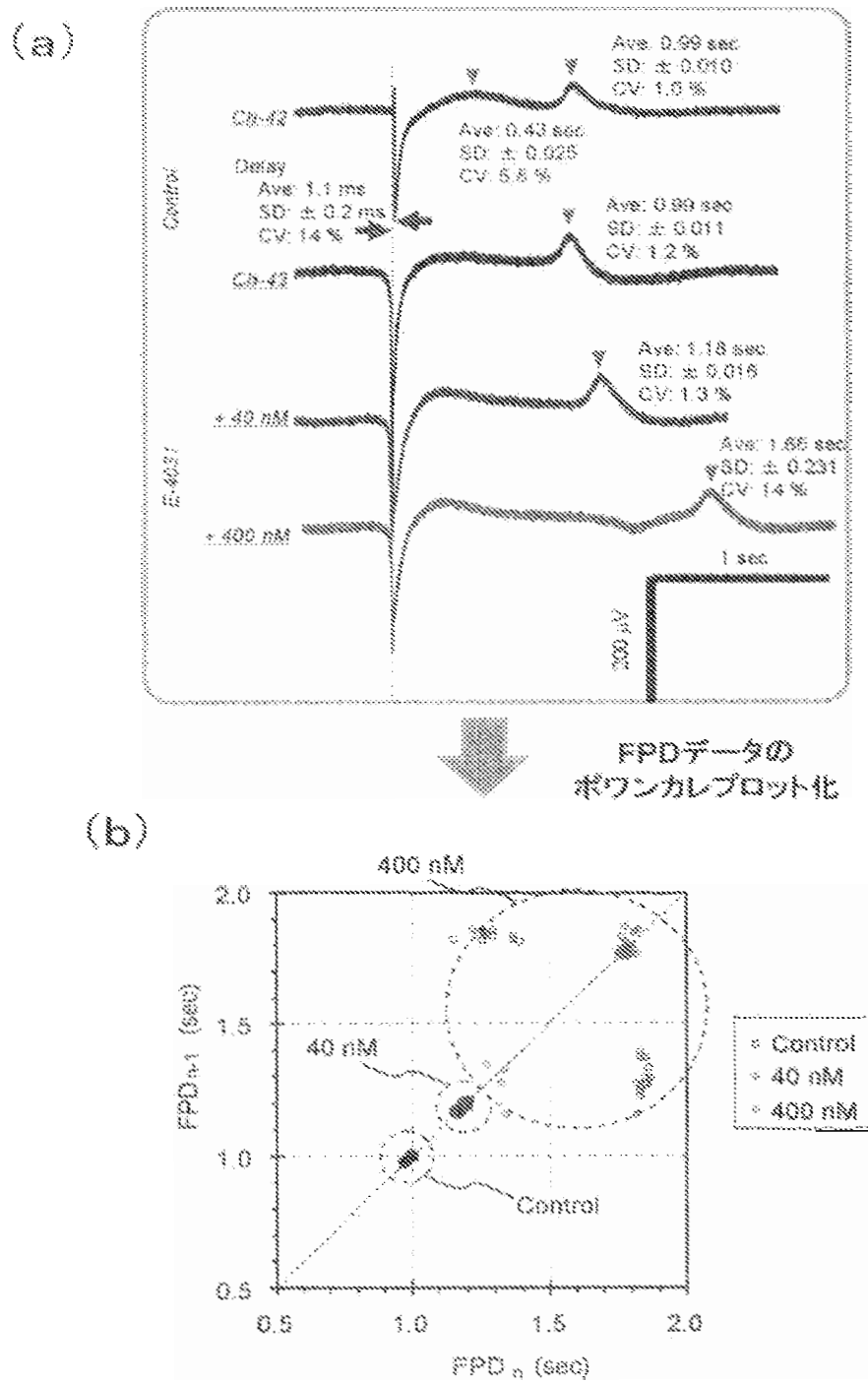


[illegible]

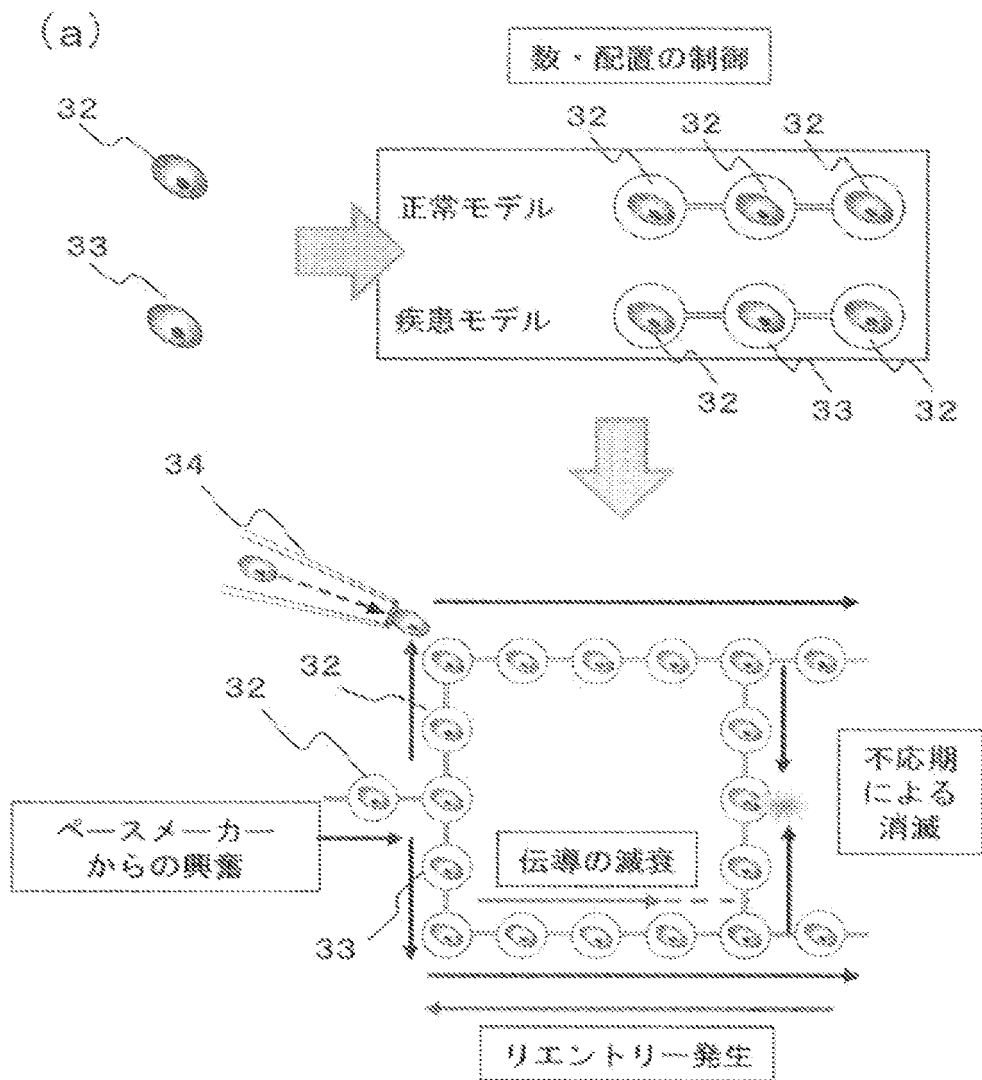
[図8]



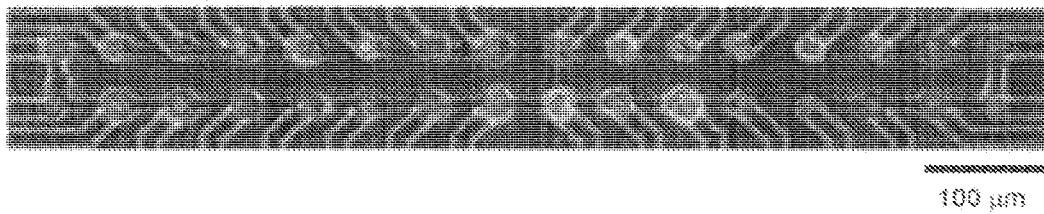
[図9]



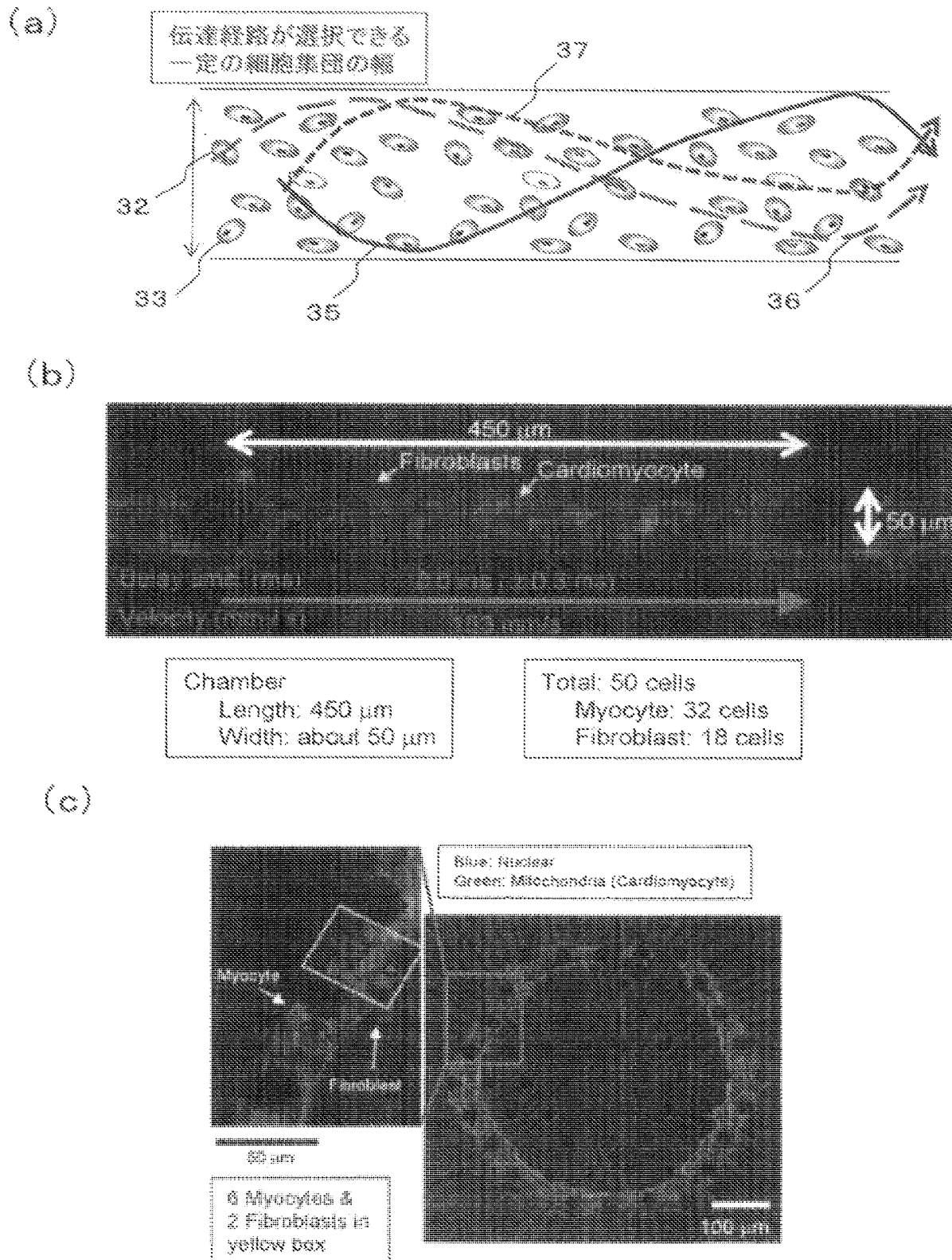
[図10]



(b)

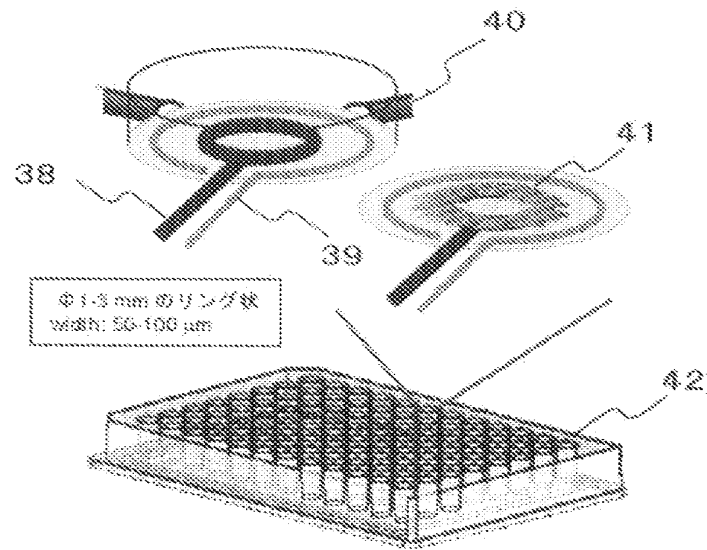


[図11]



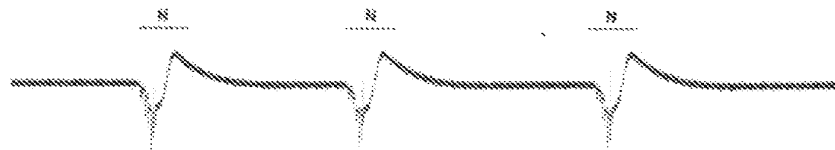
[図12]

(a)



(b)

正常伝導



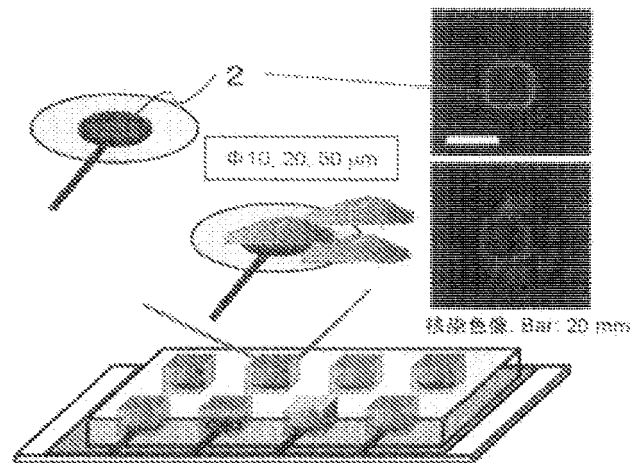
異常伝導



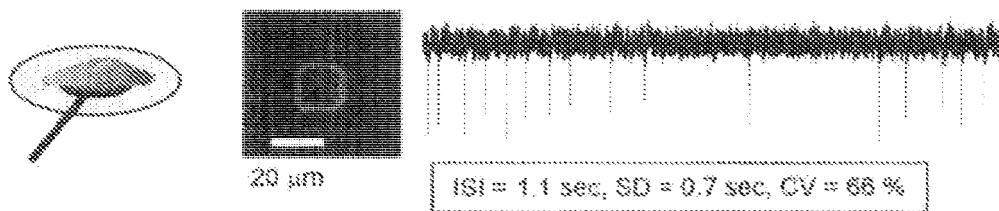
Sampling rate: ~ 1 kHz

[図13]

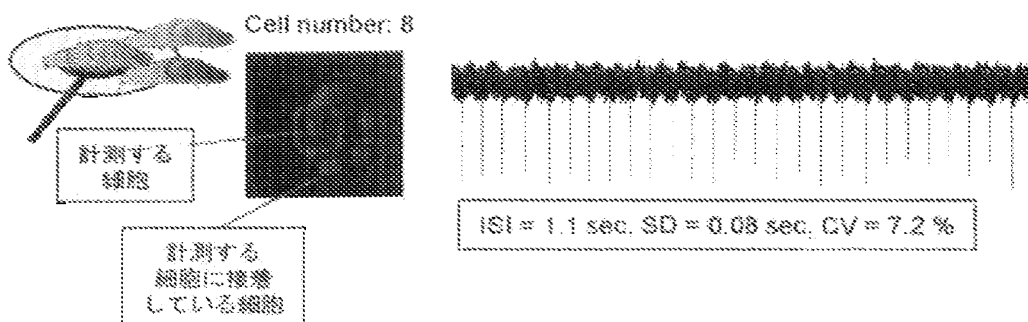
(a)



(b)

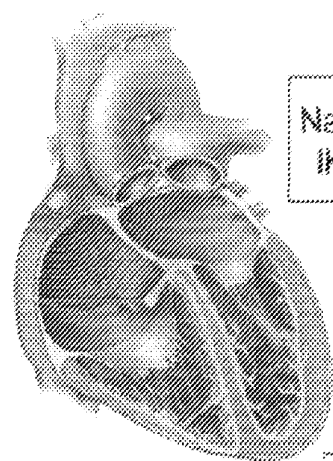


(c)



[図16]

In vivo
(個体レベル)



Na⁺, Ca²⁺ 阻害
IKr, IKs



Na⁺, Ca²⁺
IKr, IKs
電位計測
(細胞電位変化の
波形解析)

Na⁺ 阻害



電位計測
(隣接細胞間の伝達速度)

心拍波形
(不整脈)



光学計測
(心筋細胞形状変化)

血流排出量

TdP (Torsades de Pointes)
心房細動・心室細動モデル



心筋細胞ネットワークモデル
(リエントリー回路)

心肥大モデル

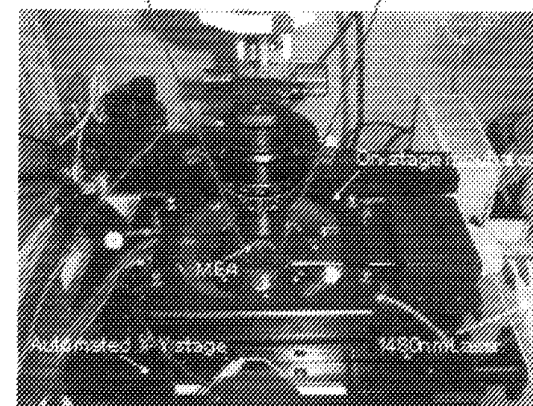


心筋細胞ネットワークモデル
(構成的細胞配置: 心筋細胞+線維細胞)

In vitro
(細胞ネットワーク)

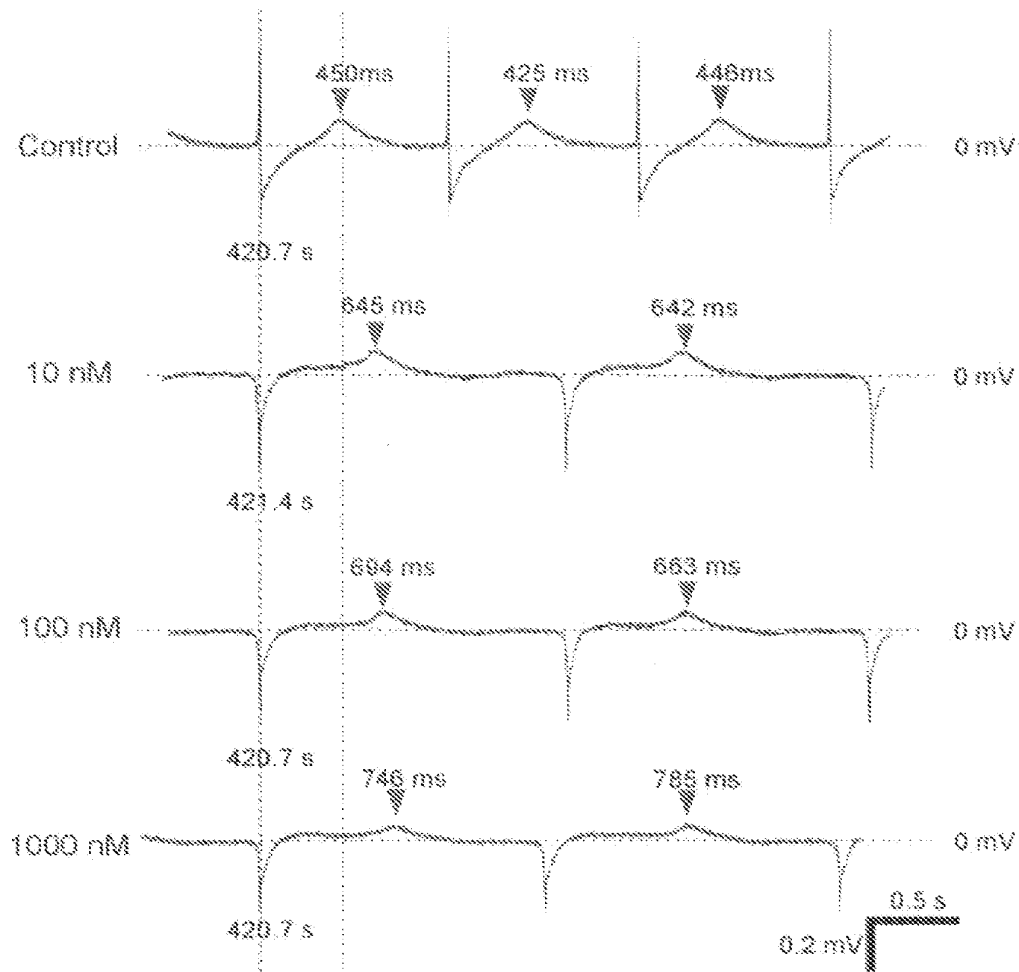


Bar: 100 μm

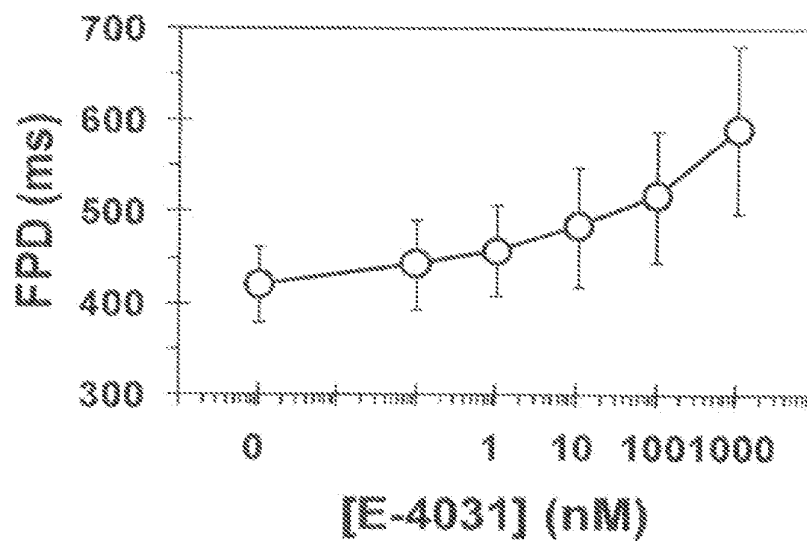


オンチップ心筋細胞ネットワーク
光学・電位計測システム

[図17]

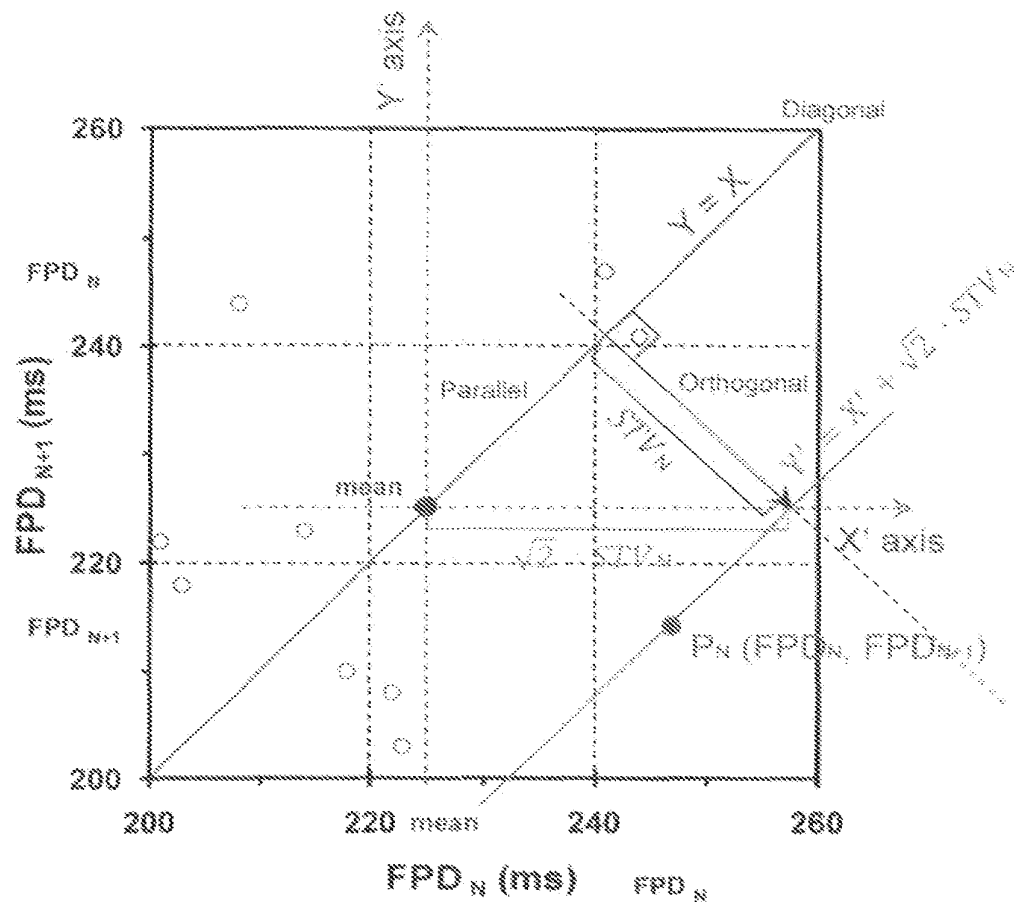


[図18]



[図19]

(a)

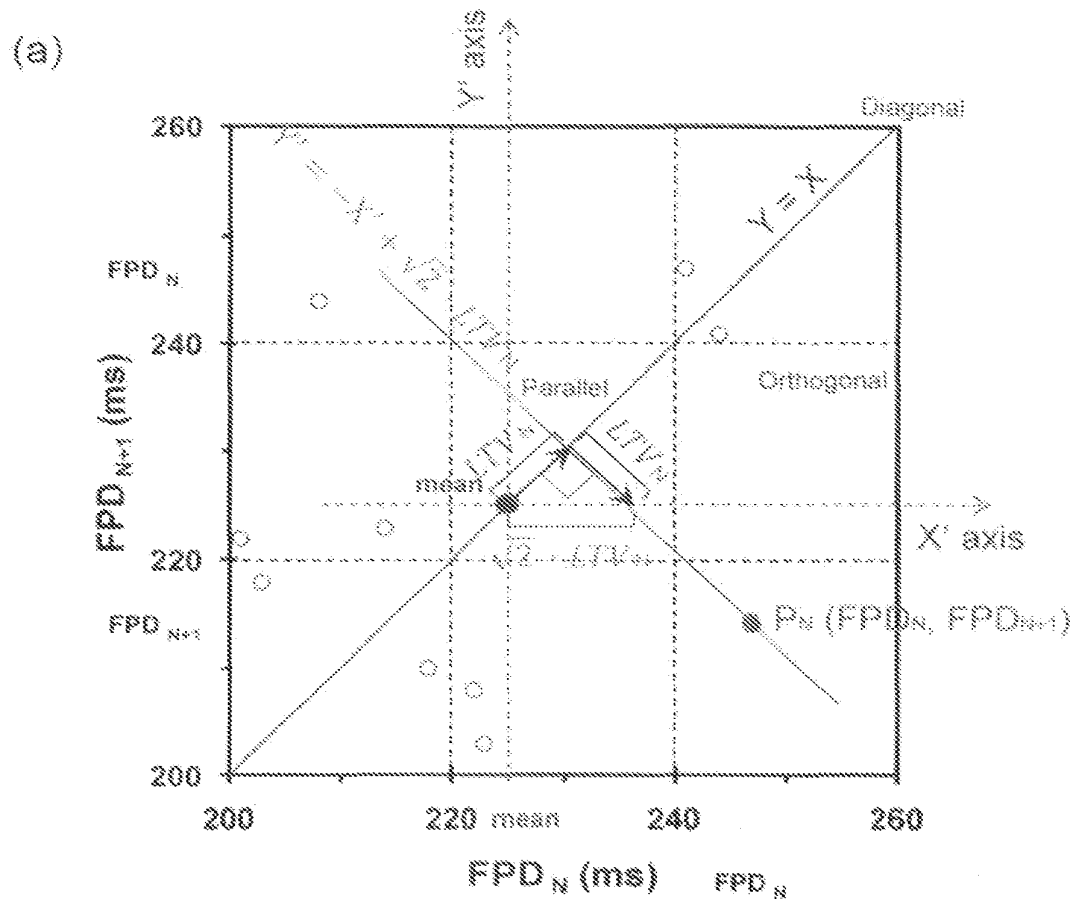


(b)

$$STV = \frac{\sum_{n=1}^k |(FPD_{n+1} - FPD_{mean}) - (FPD_n - FPD_{mean})|}{k \times \sqrt{2}}$$

$$= \frac{\sum_{n=1}^k |FPD_{n+1} - FPD_n|}{k \times \sqrt{2}} \quad \dots (式1)$$

[図20]



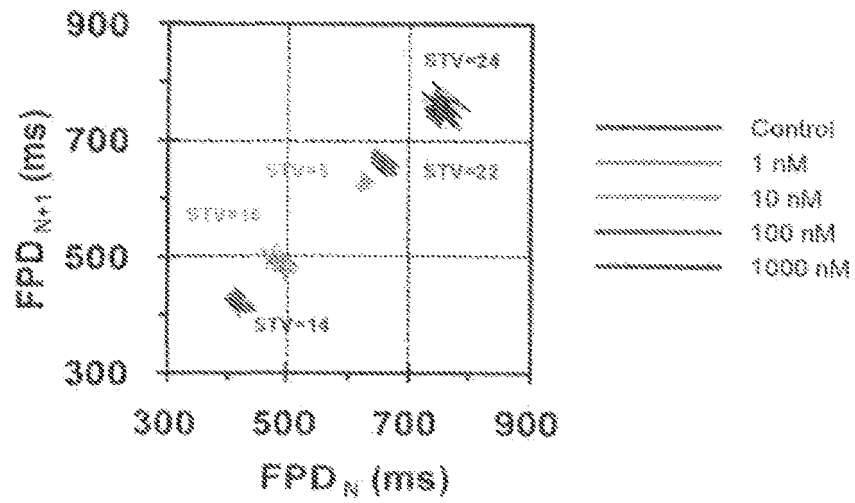
(b)

$$LTV = \frac{\sum_{n=1}^k |(FPD_{n+1} - FPD_{mean}) + (FPD_n - FPD_{mean})|}{k \times \sqrt{2}}$$

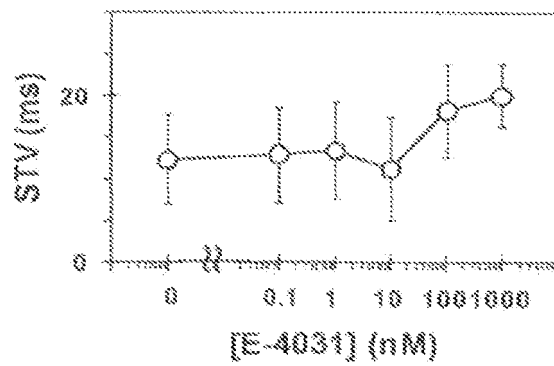
$$= \frac{\sum_{n=1}^k |FPD_{n+1} + FPD_n - 2FPD_{mean}|}{k \times \sqrt{2}} \quad \dots (式2)$$

[図21]

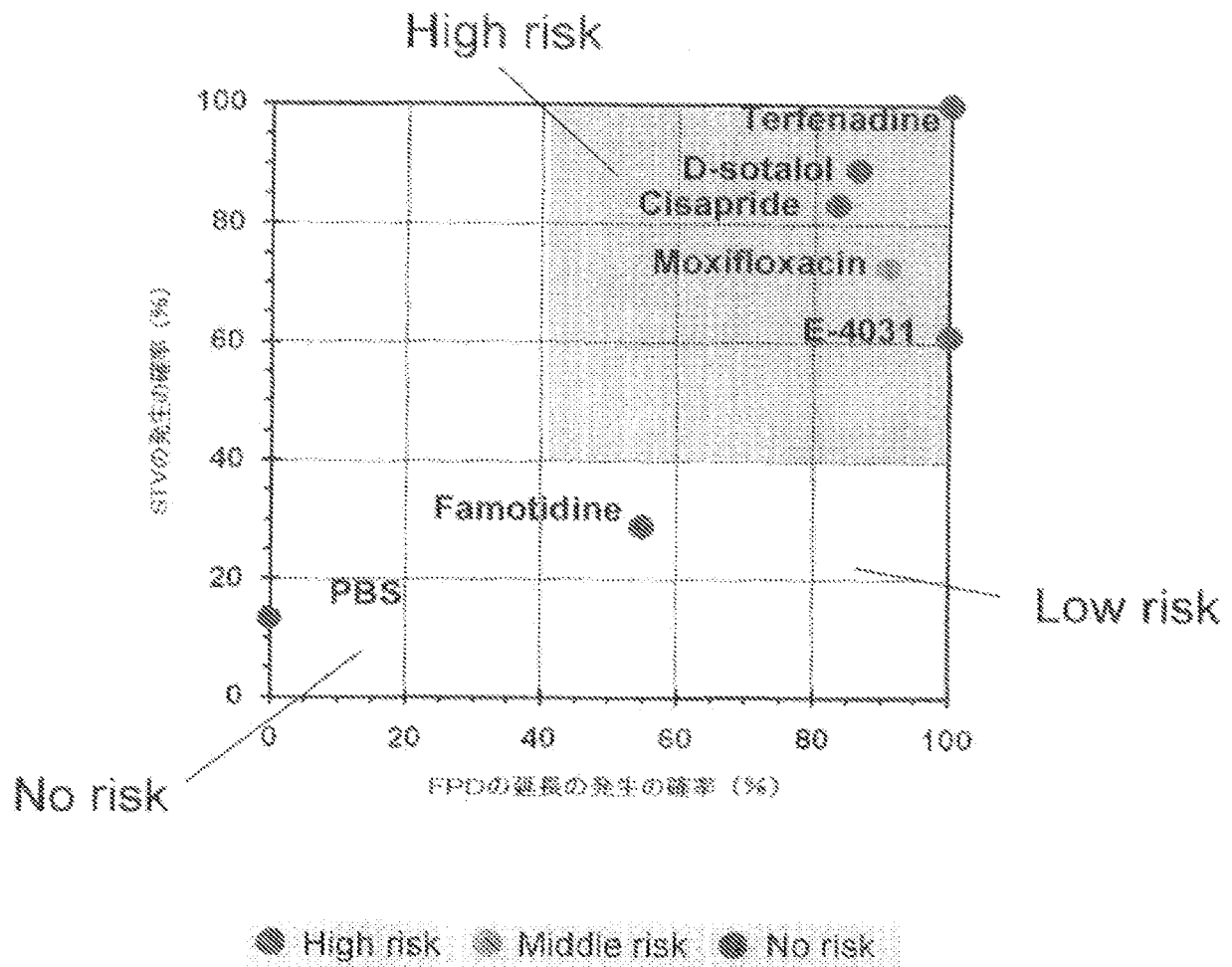
(a)



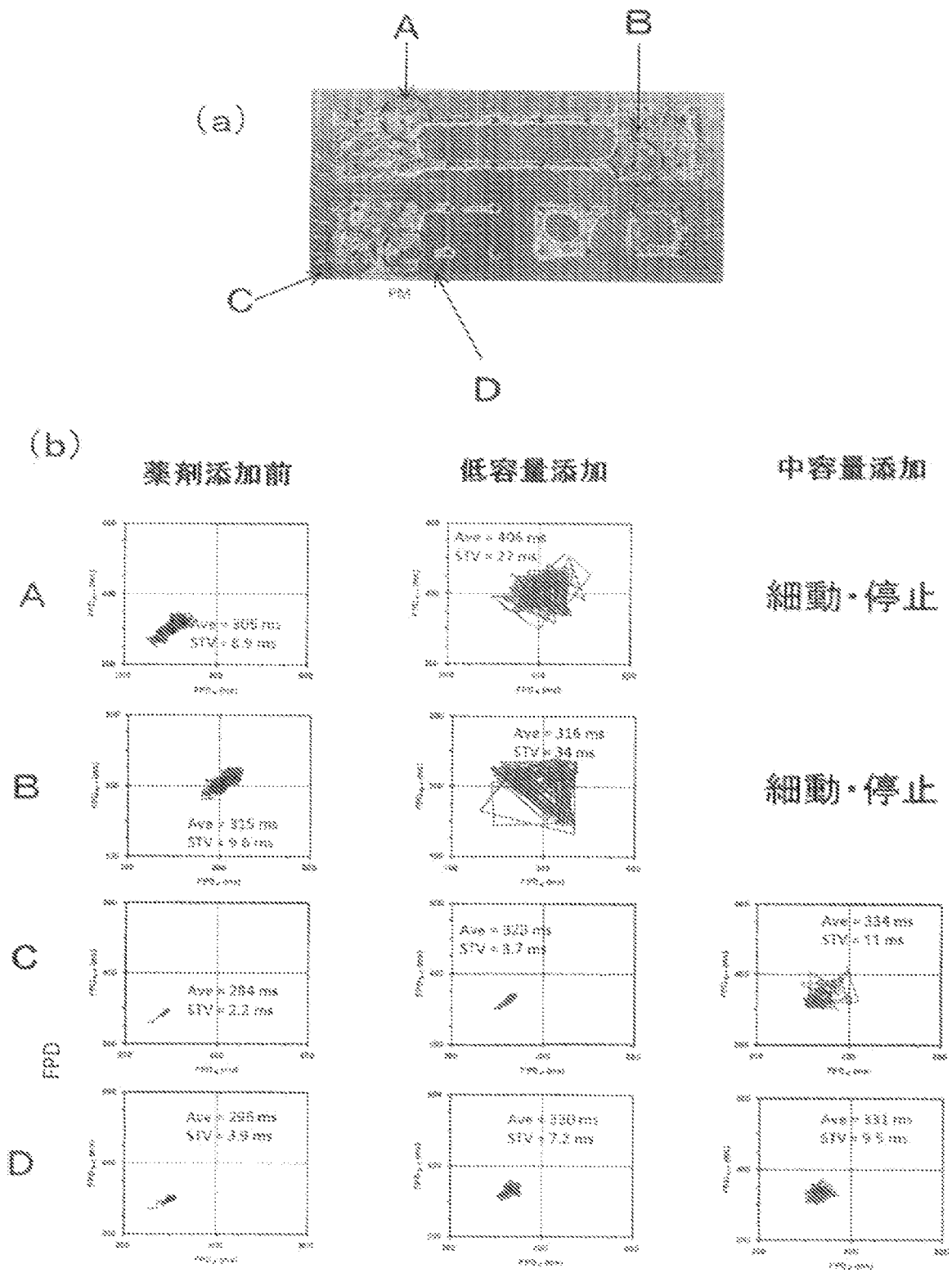
(b)



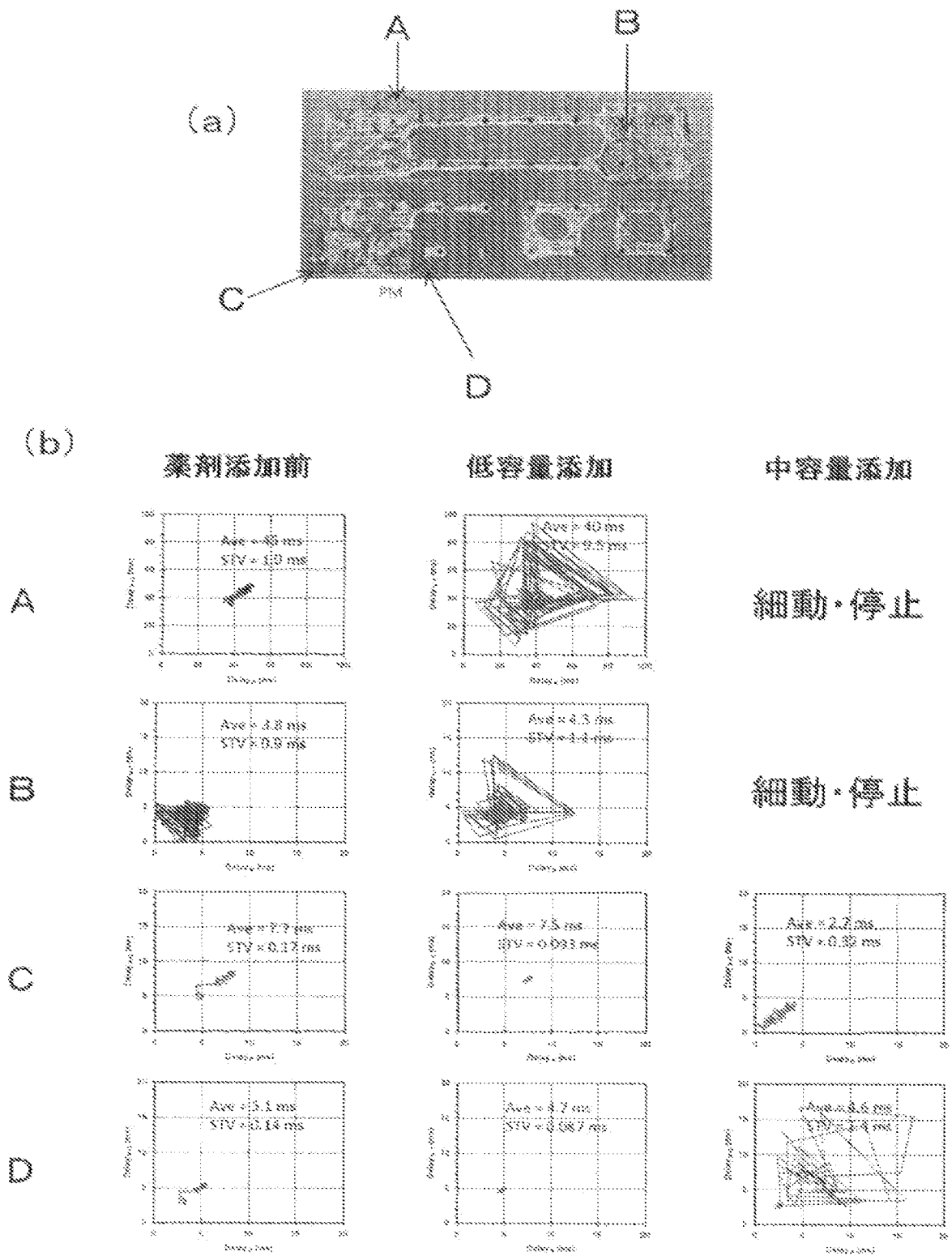
[図22]



[図23]



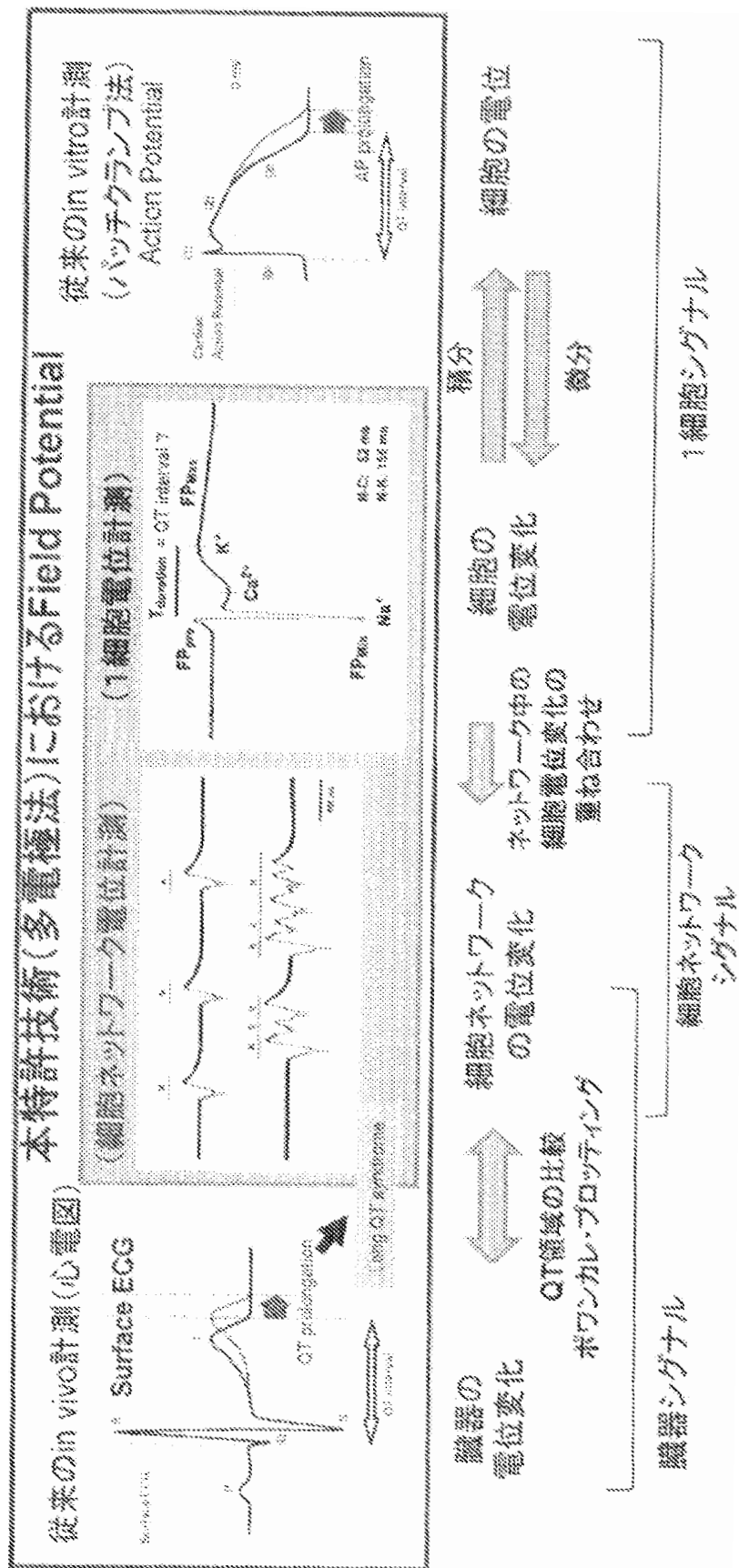
[図24]



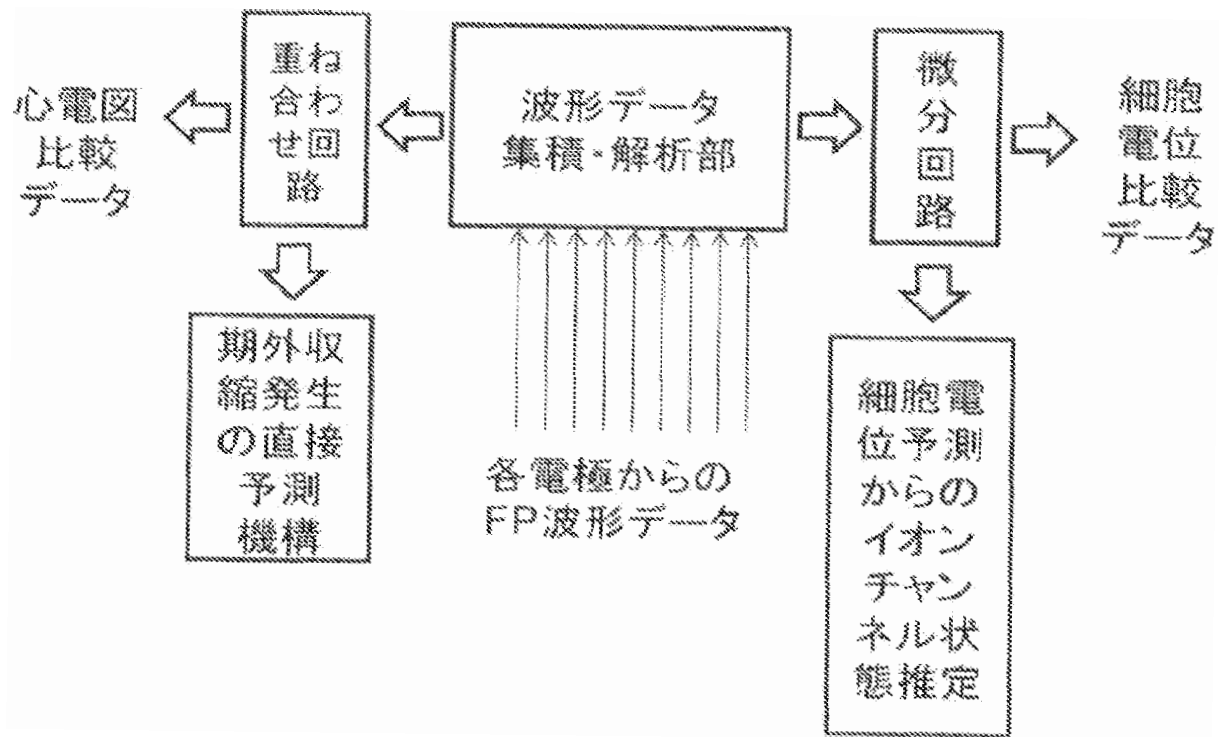
(c)

$$STV = \frac{\sum_{n=1}^k |V_{n+1} - V_n|}{k \times \sqrt{2}} \quad \dots (式3)$$

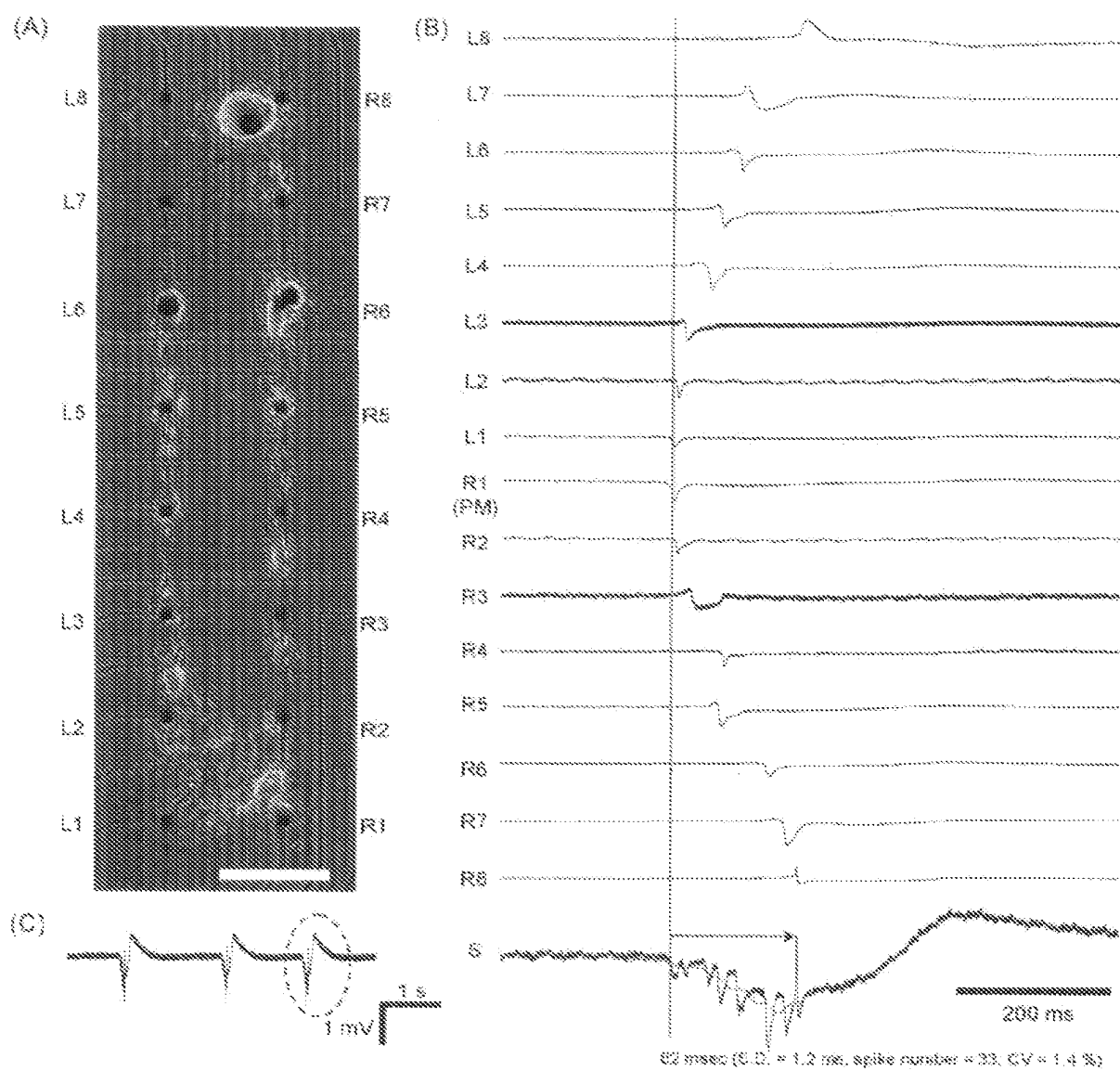
[図25]



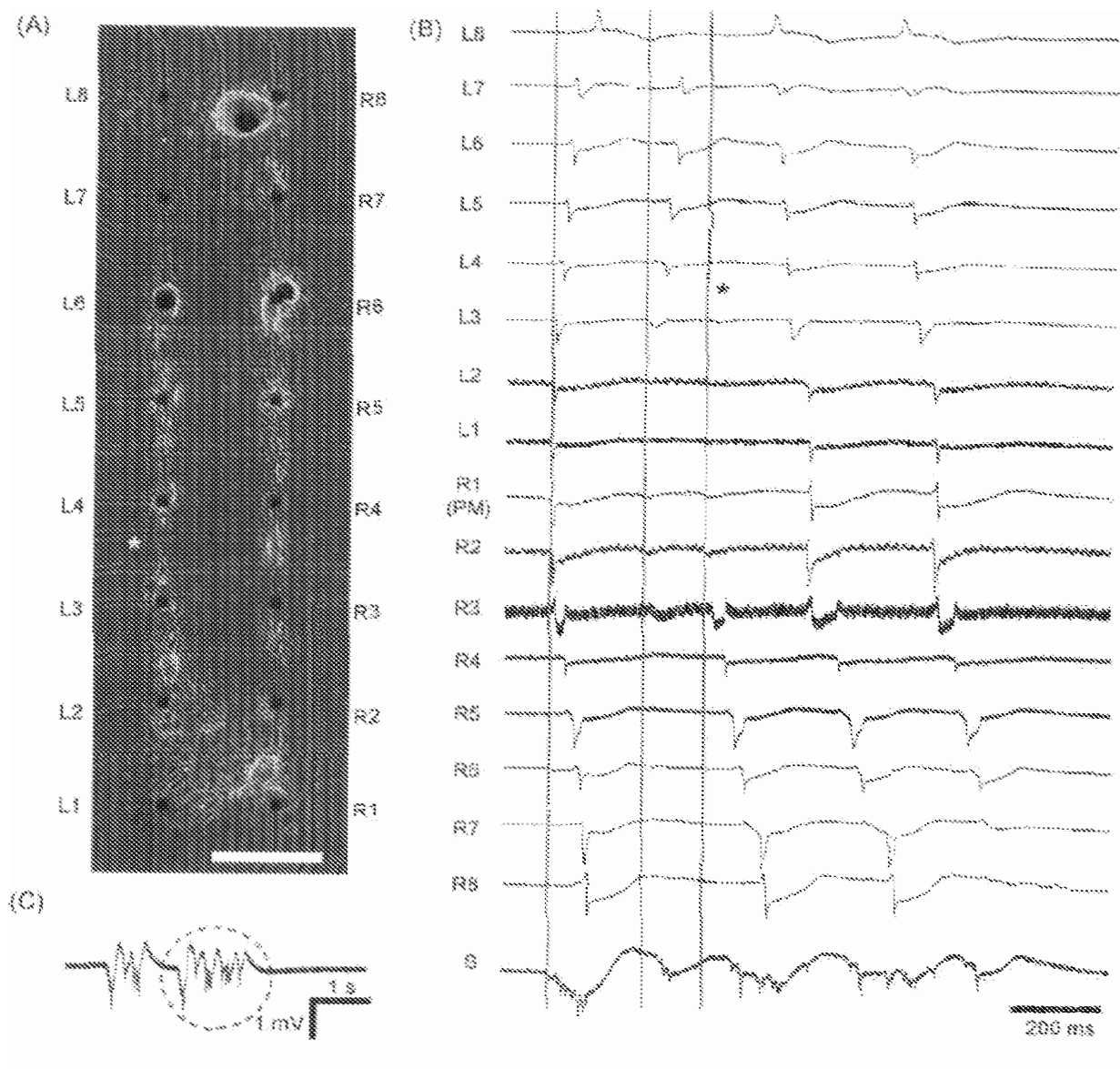
[図26]



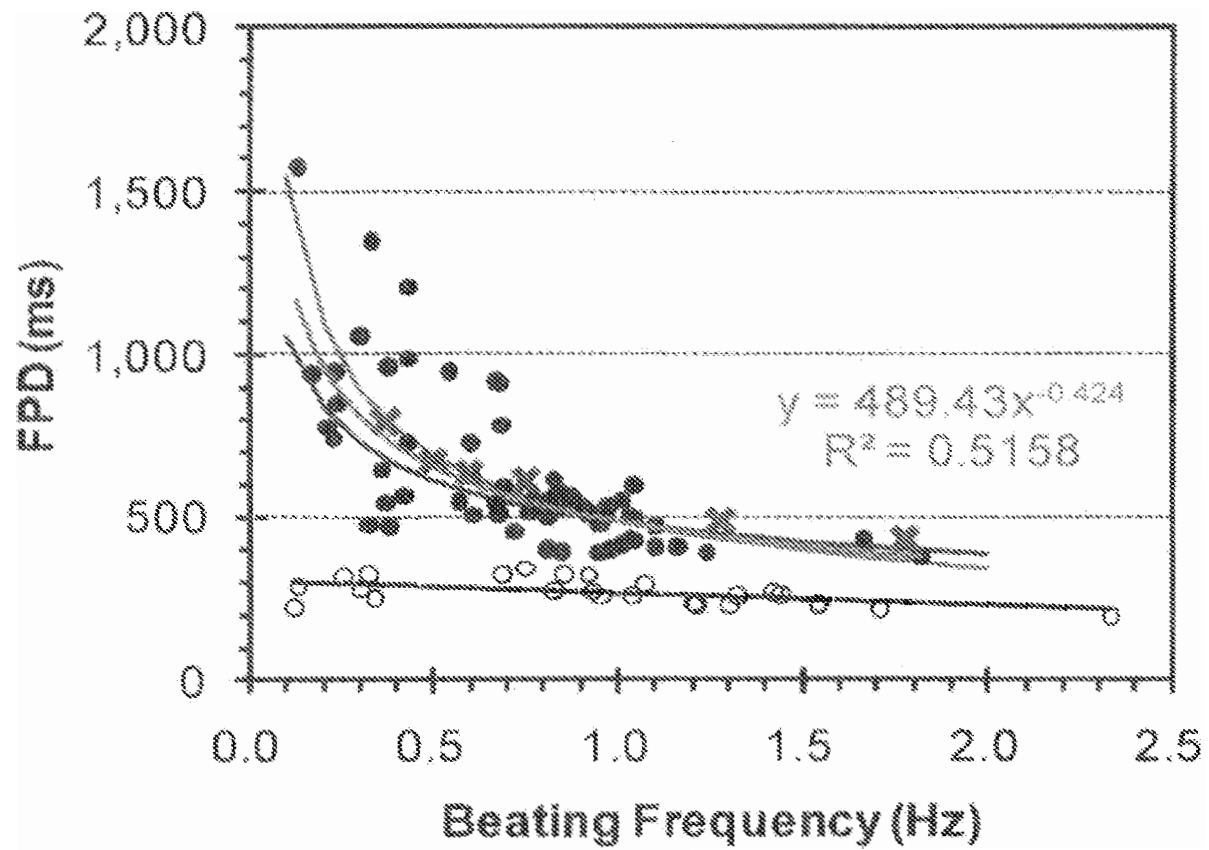
[図27]



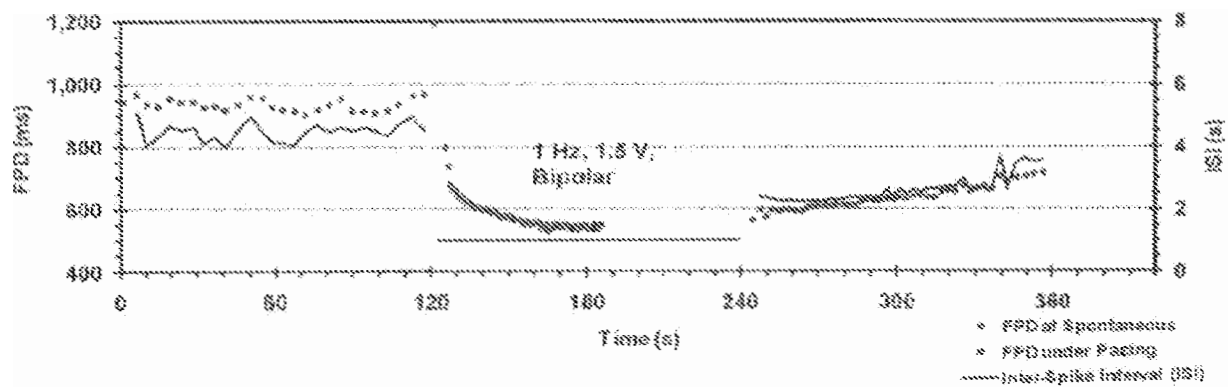
[図28]



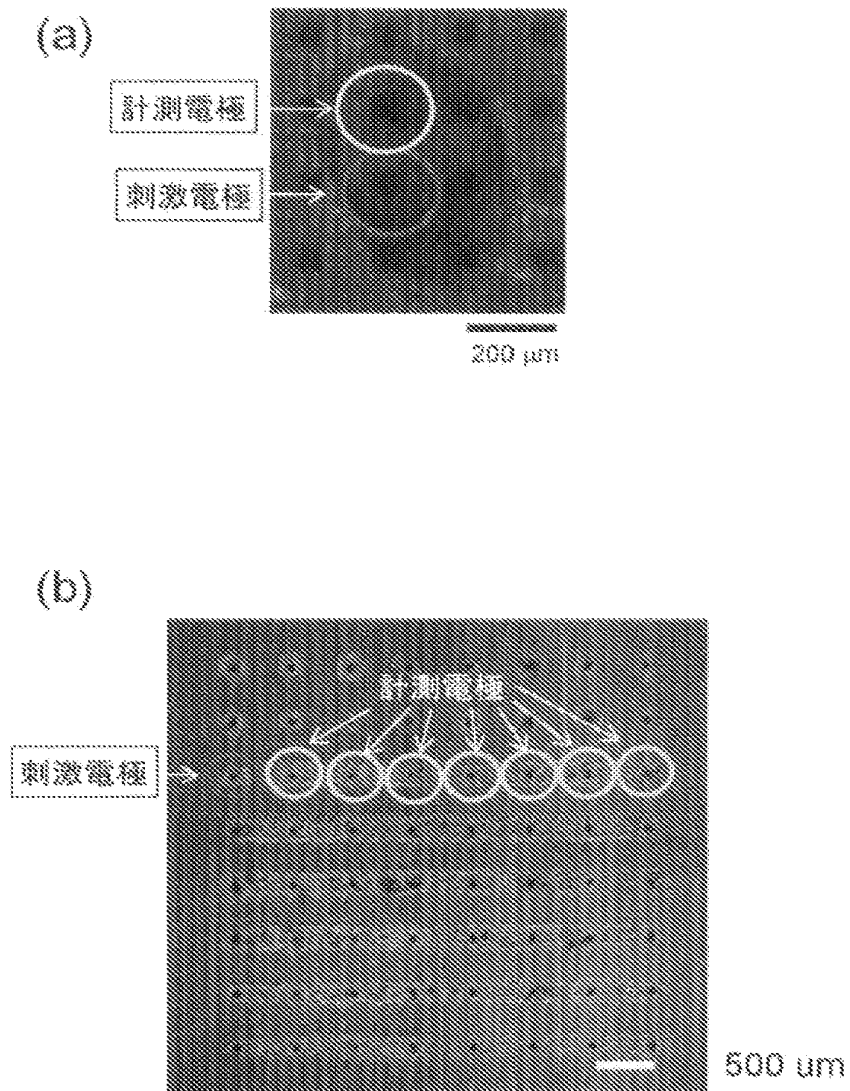
[図29]



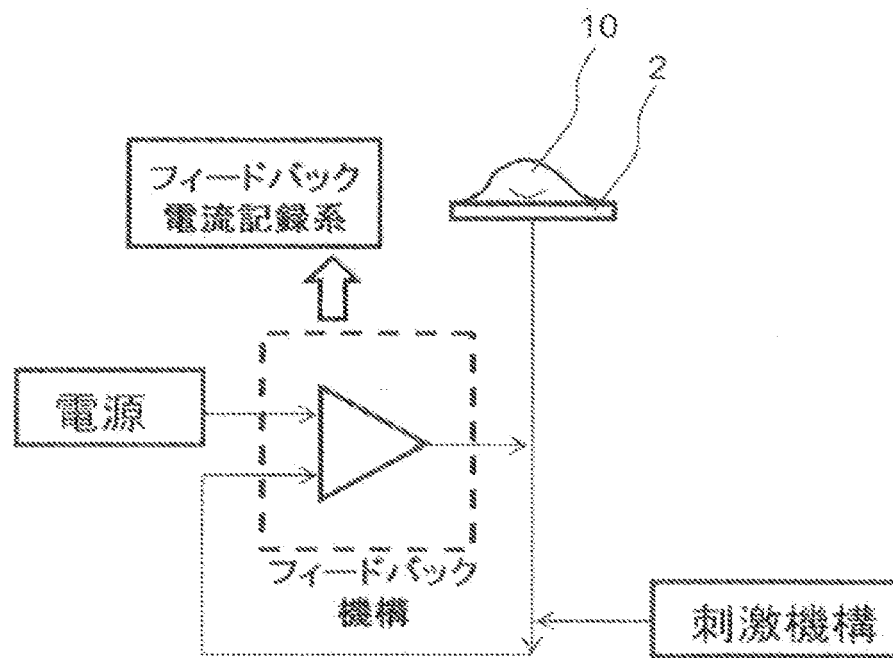
[図30]



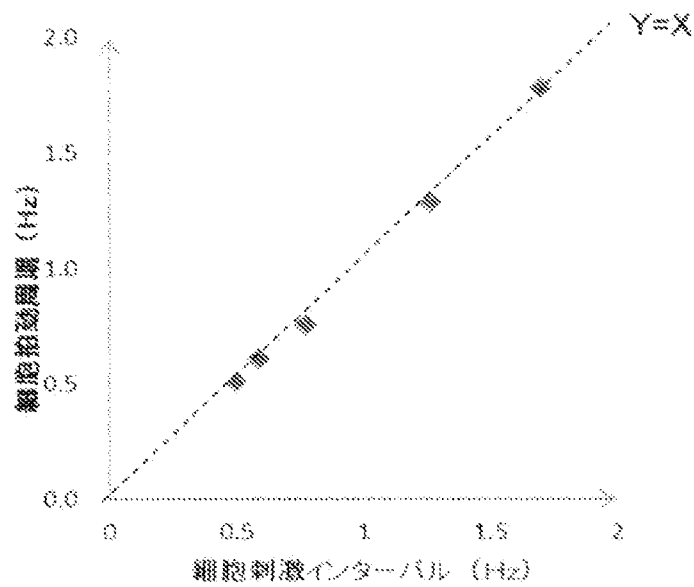
[図31]



[図32]

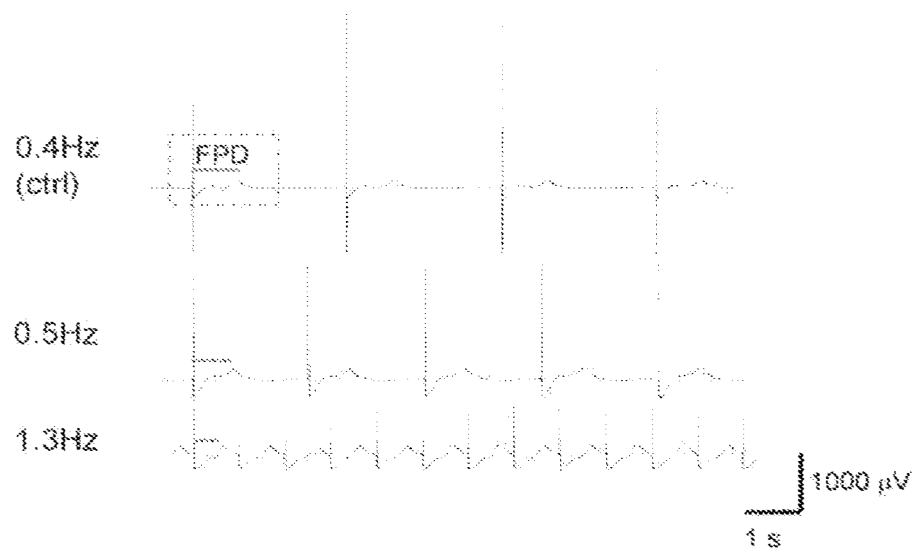


[図33]

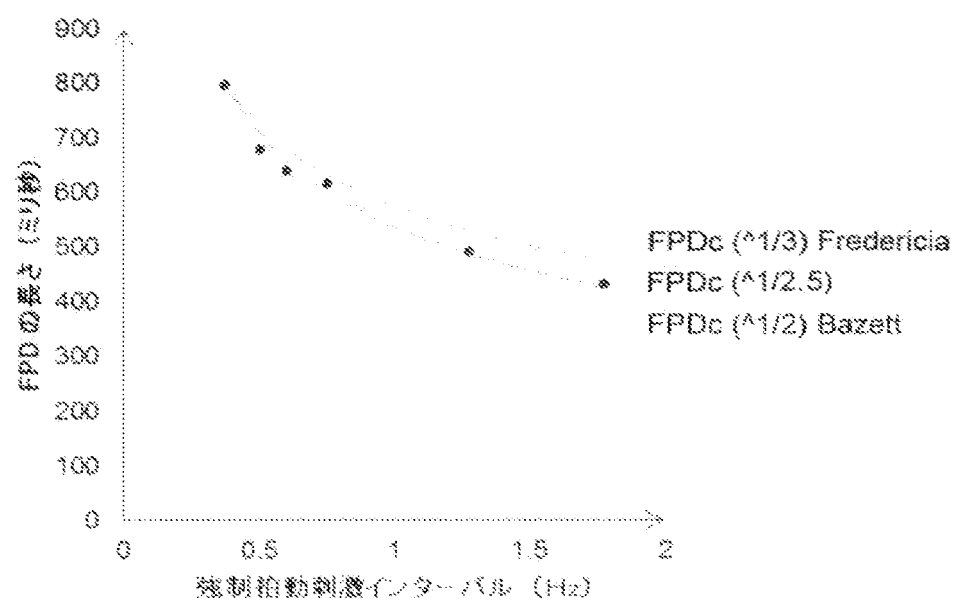


[図34]

(a)



(b)

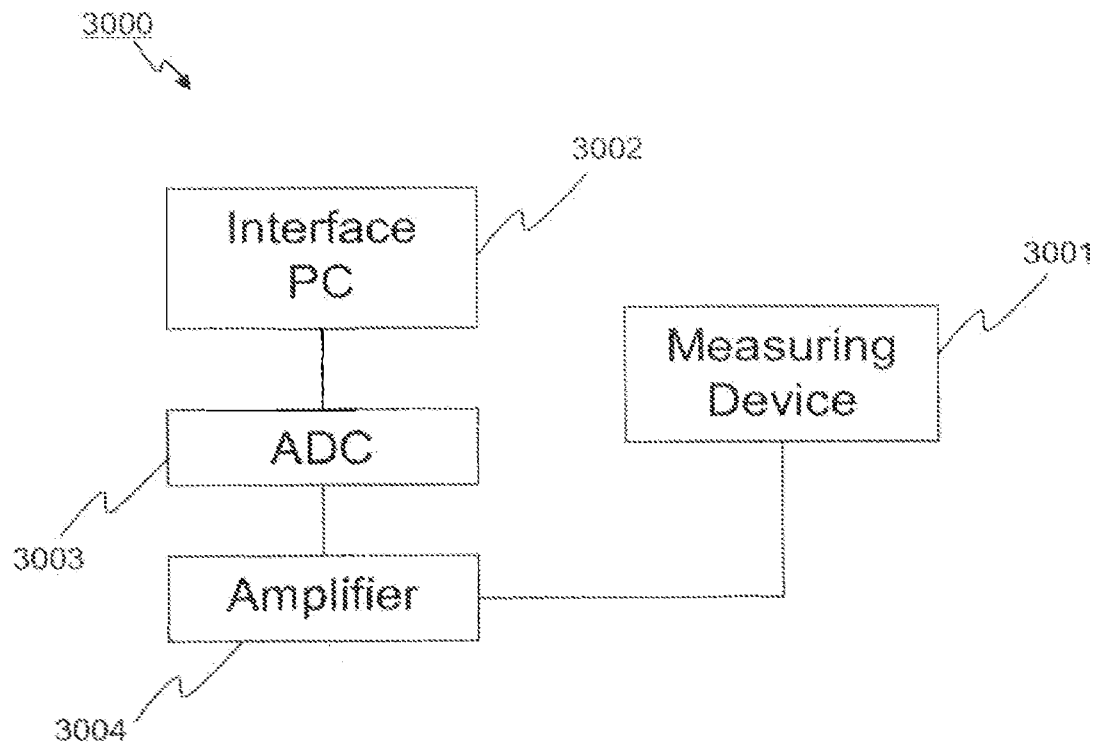


[図35]

Table. 1 Heart rate correction of beating frequency, field potential duration

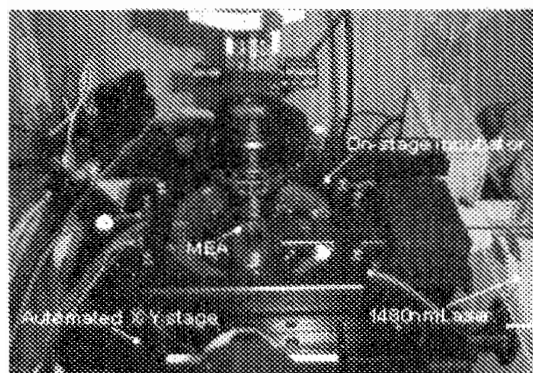
	Beating Frequency				FPD			FPDc		
	Average (Hz)	S.D. (Hz)	C.V. (%)	Spike No.	Average (ms)	S.D. (ms)	C.V. (%)	Average (ms)	S.D. (ms)	C.V. (%)
0.4 Hz (ctrl)	0.37	0.01	1.5	110	795.3	13.9	1.7	536.7	9.7	1.8
0.5 Hz	0.50	0.00	0.0	94	675.6	6.6	1.0	513.7	5.0	1.0
0.6 Hz	0.60	0.00	0.0	142	638.5	7.5	1.2	521.8	6.1	1.2
0.8 Hz	0.75	0.00	0.0	189	613.7	8.8	1.4	547.8	7.7	1.4
1.3 Hz	1.28	0.00	0.0	253	489.4	11.5	2.4	540.2	12.7	2.4
1.8 Hz	1.78	0.00	0.0	306	431.2	9.1	2.1	542.8	11.4	2.1

[図36]

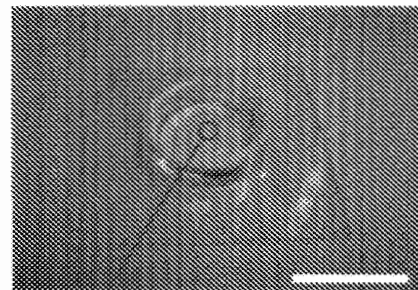


[図37]

A. Core of the system

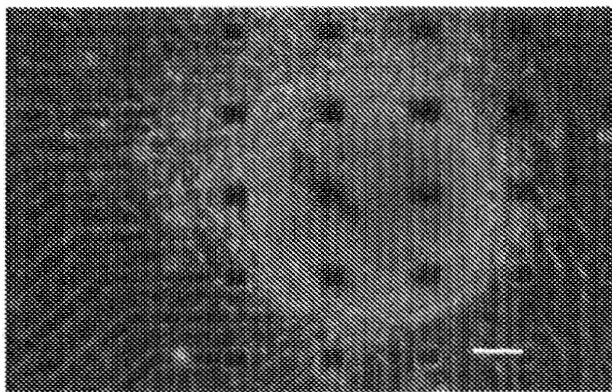


B. MEA Chip



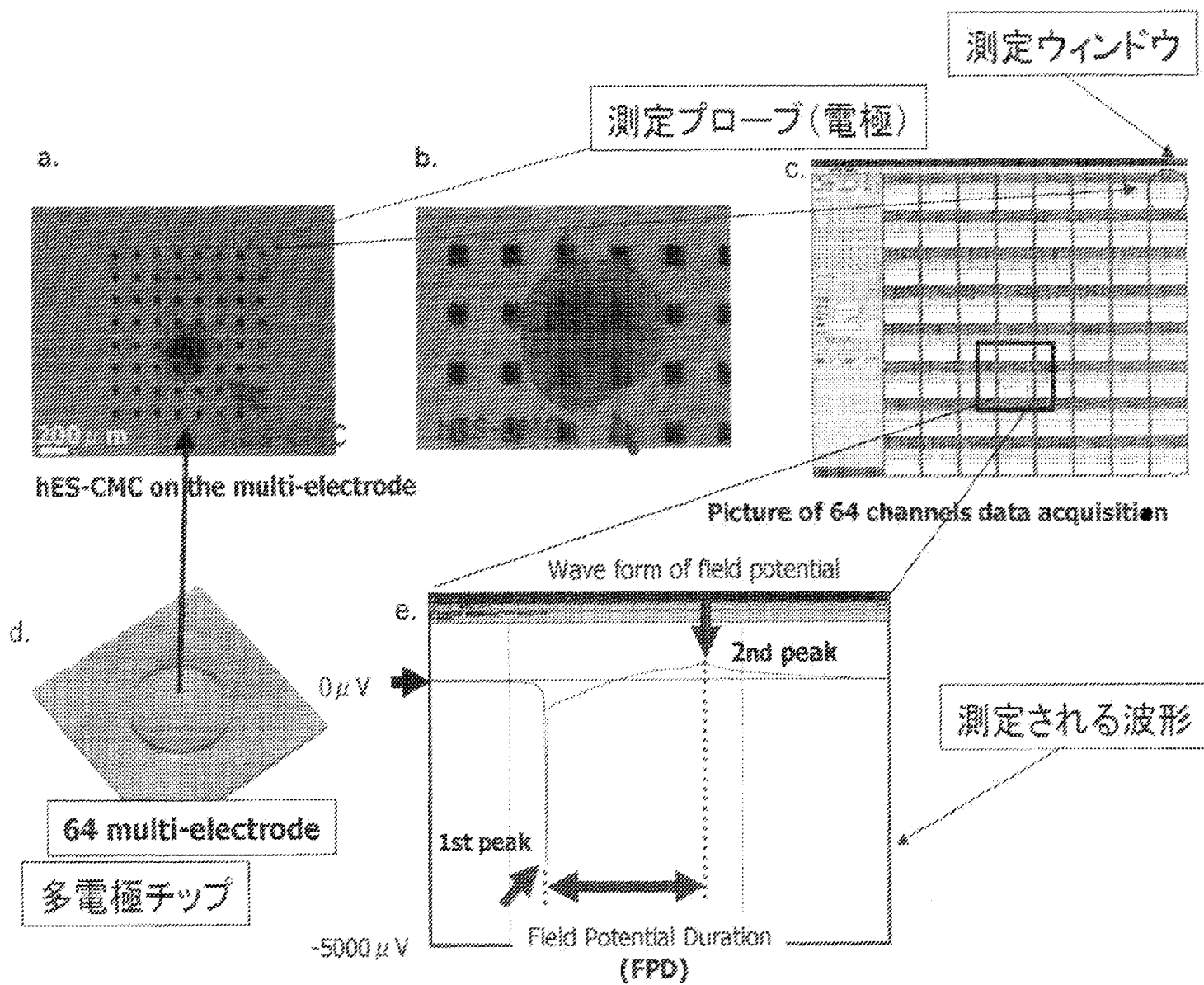
2 cm

C. hES CM on MEA Chip

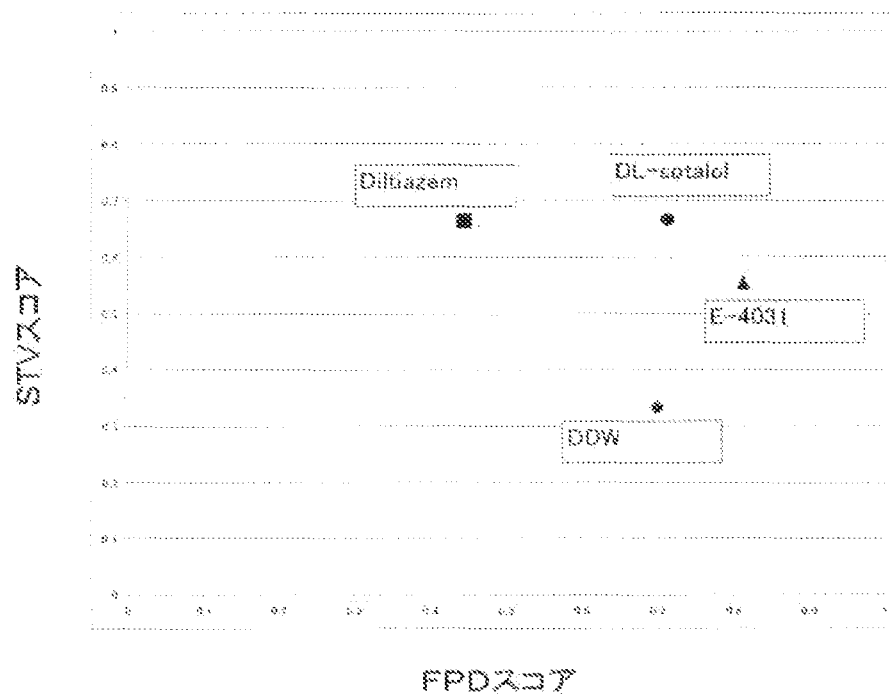


100um

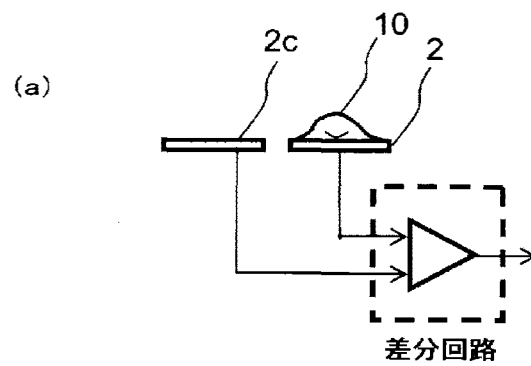
【図38】



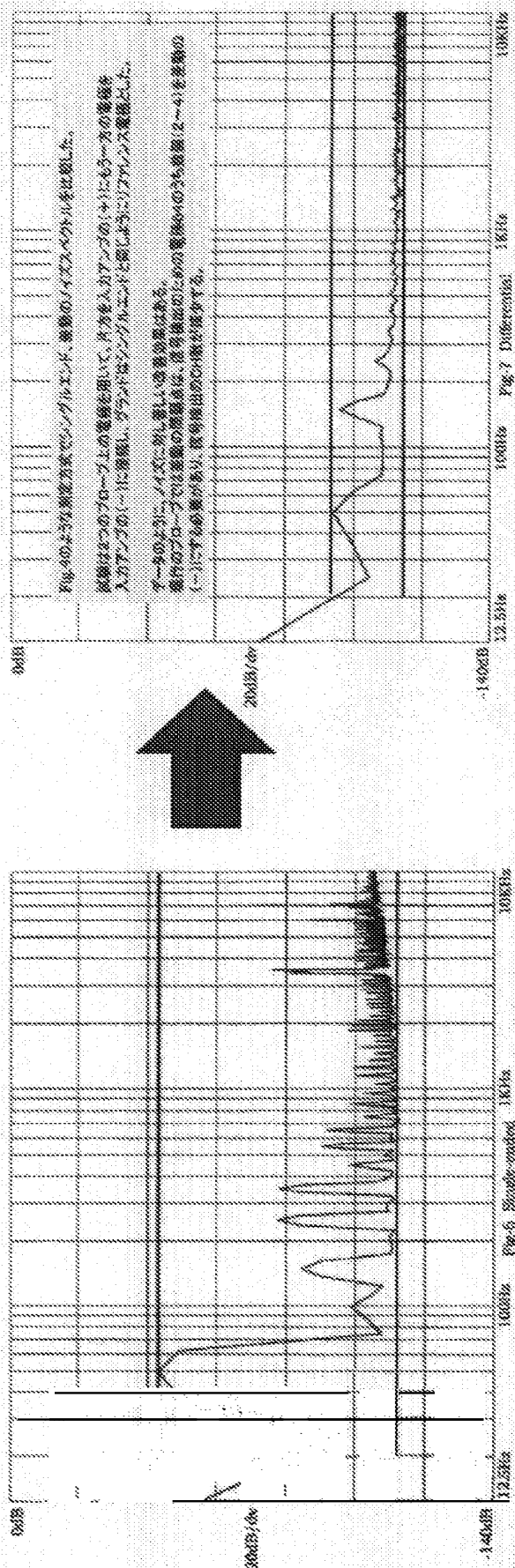
[図39]



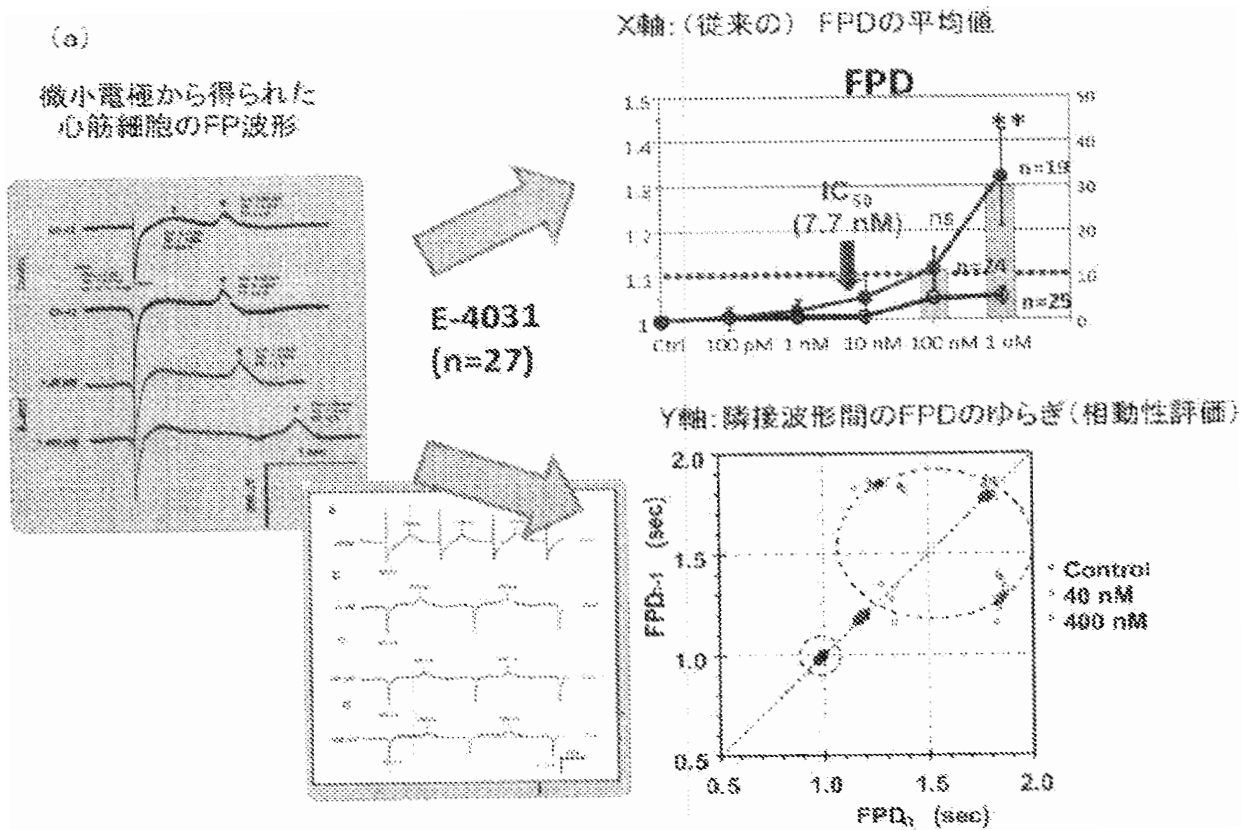
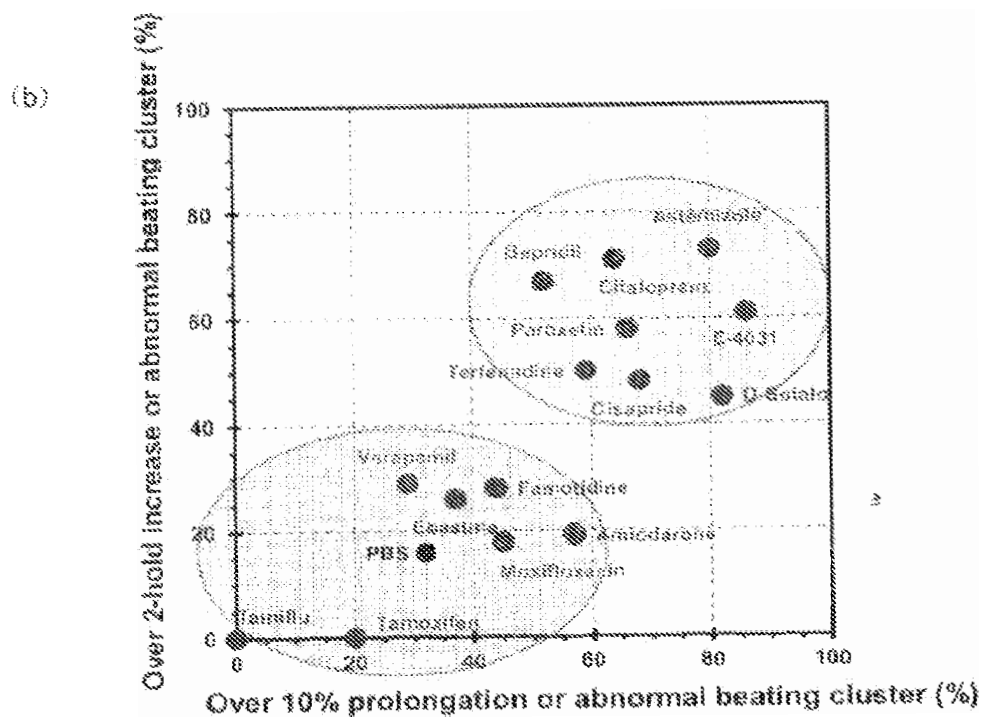
[図40a]



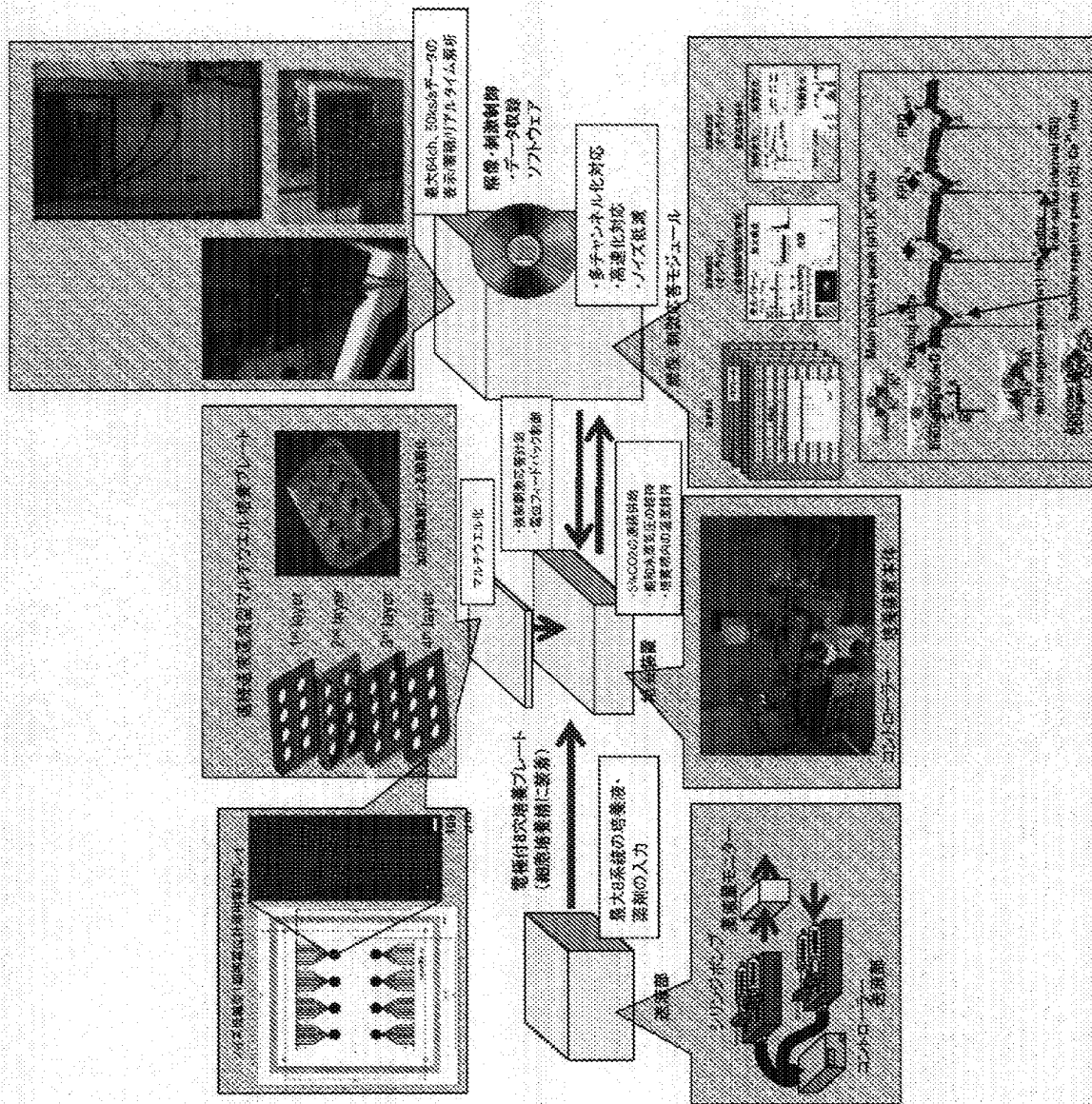
[図40c]



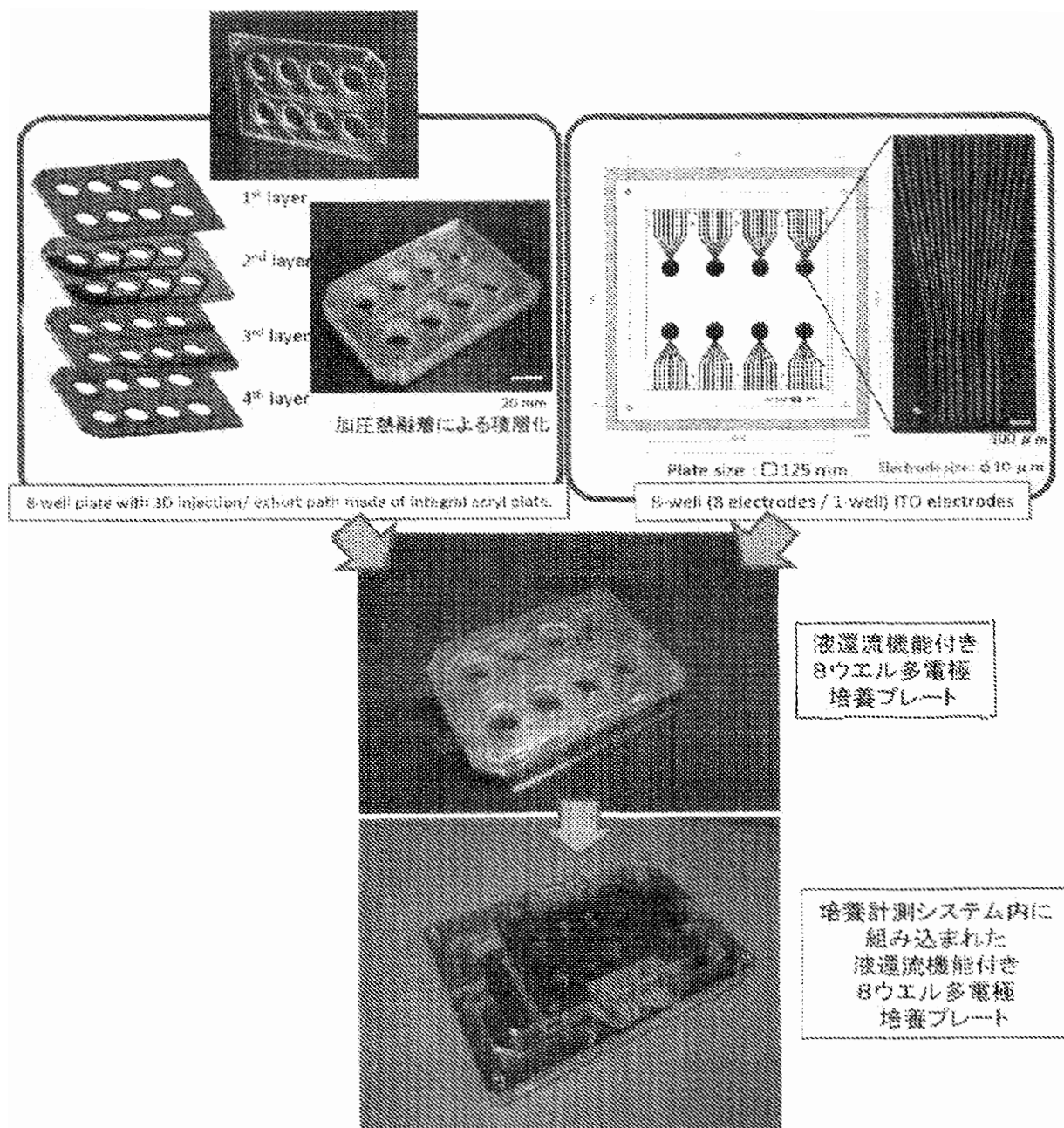
[図41]



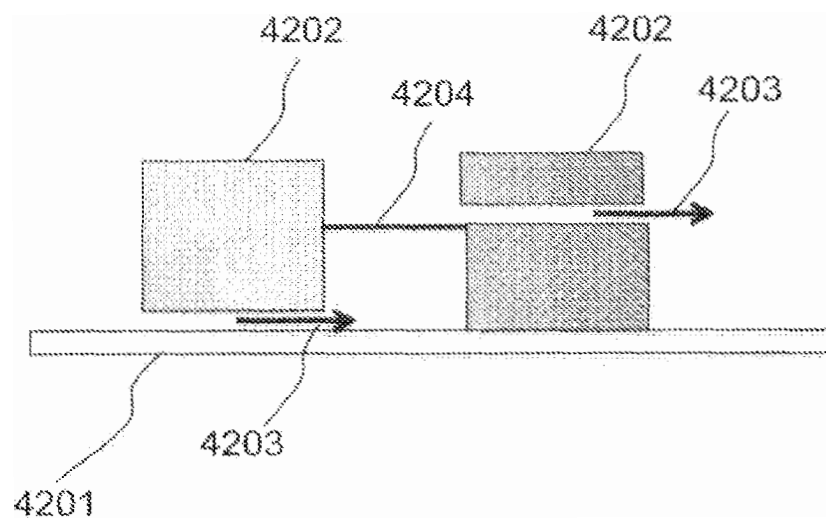
[図42]



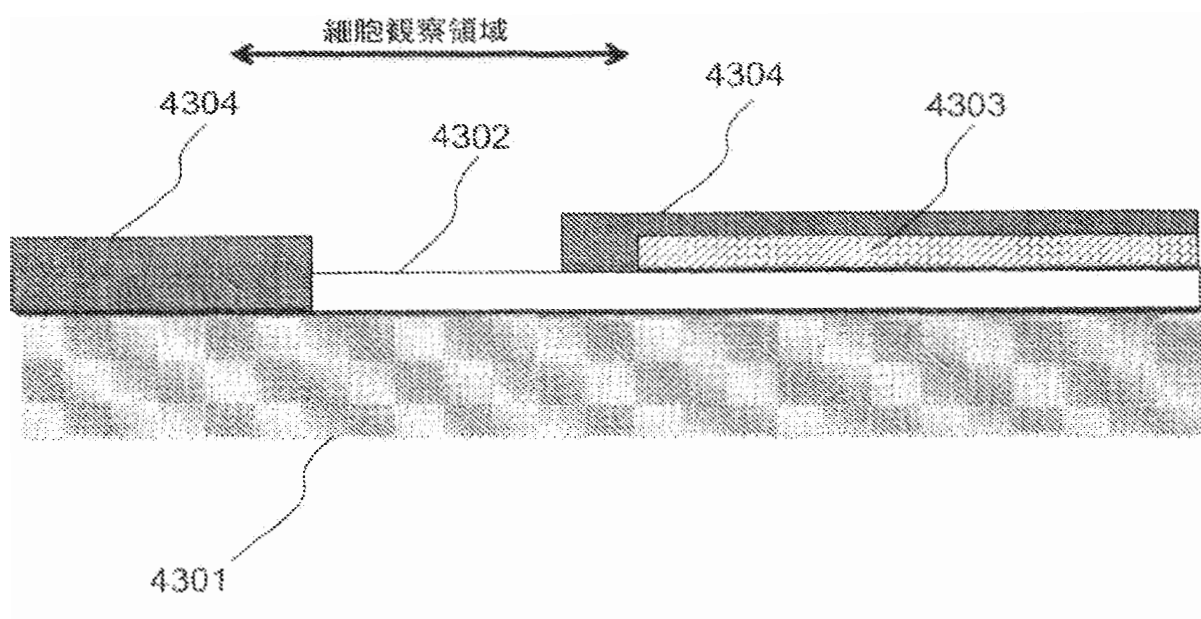
[図43]



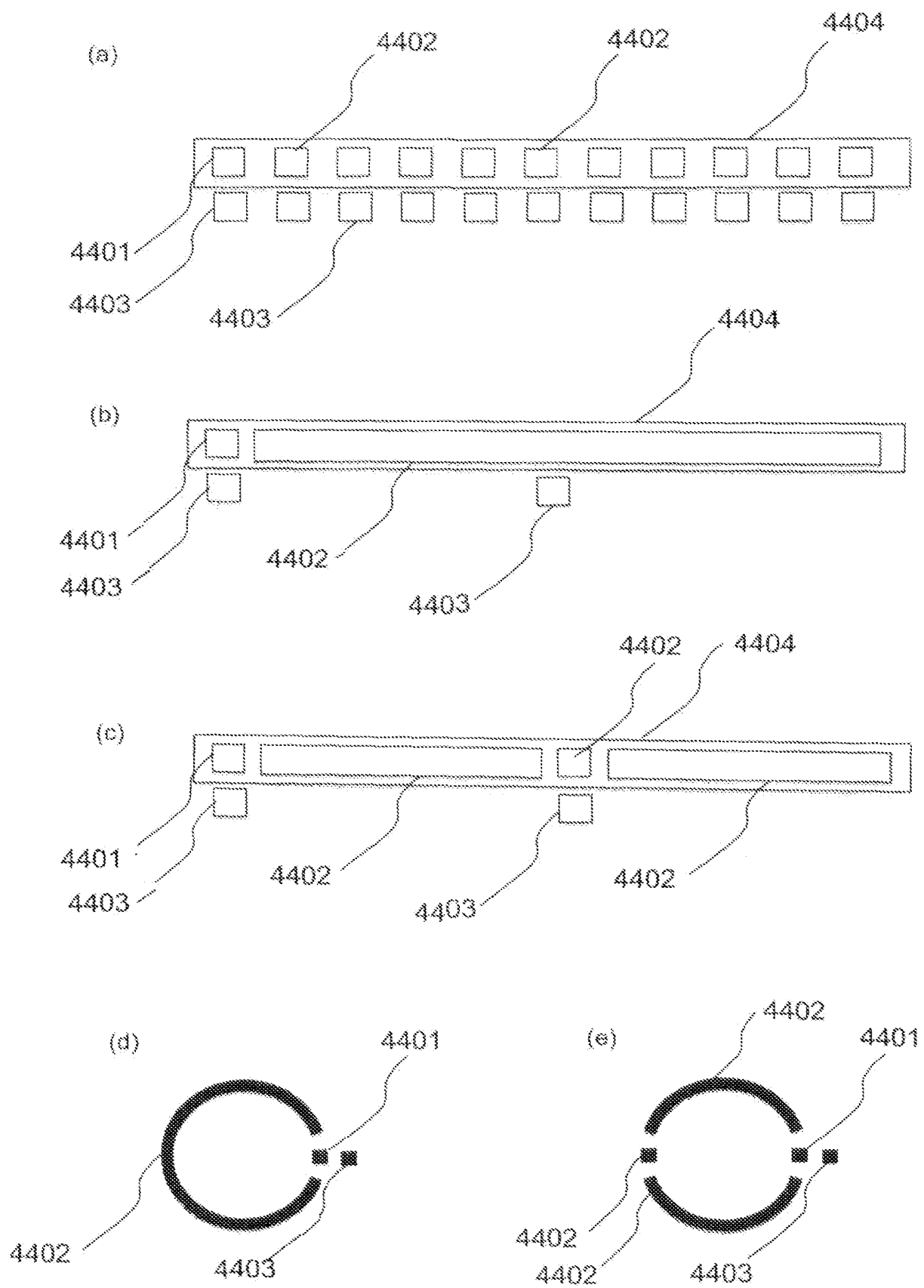
[図44]



[図45]



[図46]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/072618

A. CLASSIFICATION OF SUBJECT MATTER

C12M1/34(2006.01)i, C12Q1/02(2006.01)i

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)

C12M1/34, C12Q1/02

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

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2011
Kokai Jitsuyo Shinan Koho	1971-2011	Toroku Jitsuyo Shinan Koho	1994-2011

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

JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009/038079 A1 (Biotec Co., Ltd.), 26 March 2009 (26.03.2009), & US 2010/0304423 A1 & EP 2213721 A1	1, 2
A	JP 2006-094703 A (Onchip Cellomics Consortium), 13 April 2006 (13.04.2006), & US 2007/0059763 A1 & US 2009/0042200 A1 & US 2009/0042739 A1 & EP 1626278 A2 & EP 1901067 A2	1, 2
A	JP 2002-153260 A (Japan Science and Technology Corp.), 28 May 2002 (28.05.2002), & US 2004/0067482 A1 & EP 1344817 A1 & WO 2002/042411 A1	1, 2



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

"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

"&"

document member of the same patent family

Date of the actual completion of the international search

21 December, 2011 (21.12.11)

Date of mailing of the international search report

10 January, 2012 (10.01.12)

Name and mailing address of the ISA/

Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/072618

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Tsuneyoshi SAITO et al., "ES Saibo o Tsukatta Soyaku, Screening Business ni Tsuite", Gekkan Medical Science Digest, 12 Gatsu enlarged issue, 25 December 2007 (25.12.2007), vol.33, no.14, whole no.433, pages 33(1272) to 36(1275)	1, 2
A	Kenji YASUDA, "On-chip single-cell-based analysis system exploiting microfabrication technologies", Oyo Butsuri, 2005, vol.74, no.7, pages 890 to 895	1, 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/072618

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(Invention group 1) claims 1 and 2

Inventions relating to a device for evaluating cell qualities, said device having a specific structure, and a method for evaluating cell qualities using the device.

(Invention group 2) claims 3-15 and 16-20

Inventions relating to a device for examining myocardial toxicity and a method for examining myocardial toxicity, said device and said method being characterized in that the magnitude of fluctuation in a pulsating field potential waveform is measured by comparing with the adjacent pulsating signal.

(Continued to extra sheet.)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-2

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/072618

Continuation of Box No.III of continuation of first sheet(2)

Although claims 1 and 2 belonging to invention group 1 are cited in claims 14 and 15, the citation parts are excluded since "a device for examining myocardial toxicity" does not appear in invention group 1.

A. 発明の属する分野の分類 (国際特許分類 (IPC))

Int.Cl. C12M1/34(2006.01)i, C12Q1/02(2006.01)i

B. 調査を行った分野

調査を行った最小限資料 (国際特許分類 (IPC))

Int.Cl. C12M1/34, C12Q1/02

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	1922-1996年
日本国公開実用新案公報	1971-2011年
日本国実用新案登録公報	1996-2011年
日本国登録実用新案公報	1994-2011年

国際調査で使用した電子データベース (データベースの名称、調査に使用した用語)

JSTPlus/JMEDPlus/JST7580(JDreamII)

C. 関連すると認められる文献

引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求項の番号
A	WO 2009/038079 A1 (バイオテック株式会社) 2009.03.26 & US 2010/0304423 A1 & EP 2213721 A1	1,2
A	JP 2006-094703 A (有限責任中間法人 オンチップ・セロミクス・ コンソーシアム) 2006.04.13 & US 2007/0059763 A1 & US 2009/0042200 A1 & US 2009/0042739 A1 & EP 1626278 A2 & EP 1901067 A2	1,2

C欄の続きにも文献が列挙されている。

パテントファミリーに関する別紙を参照。

* 引用文献のカテゴリー

「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献 (理由を付す)
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願

の日の後に公表された文献

「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

国際調査を完了した日

21.12.2011

国際調査報告の発送日

10.01.2012

国際調査機関の名称及びあて先

日本国特許庁 (ISA/J P)

郵便番号 100-8915

東京都千代田区霞が関三丁目4番3号

特許庁審査官 (権限のある職員)

平塚 政宏

電話番号 03-3581-1101 内線 3485

4N

9041

C (続き) . 関連すると認められる文献		
引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求項の番号
A	JP 2002-153260 A (科学技術振興事業団) 2002.05.28, & US 2004/0067482 A1 & EP 1344817 A1 & WO 2002/042411 A1	1, 2
A	斉藤経義他, E S 細胞を使った創薬, スクリーニングビジネスについて, 月刊メディカル・サイエンス・ダイジェスト 1 2 月増大号, 2007.12.25, 第 3 3 巻第 1 4 号通巻 4 3 3 号, p.33(1272)-36(1275)	1, 2
A	安田賢二, 微細加工技術を用いたオンチップ細胞培養解析システム, 応用物理, 2005, 第 74 巻第 7 号, pp.890-895	1, 2

第Ⅱ欄 請求の範囲の一部の調査ができないときの意見（第1ページの2の続き）

法第8条第3項（PCT17条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

1. ☐ 請求項 _____ は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、
2. ☐ 請求項 _____ は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、
3. ☐ 請求項 _____ は、従属請求の範囲であってPCT規則6.4(a)の第2文及び第3文の規定に従って記載されていない。

第Ⅲ欄 発明の単一性が欠如しているときの意見（第1ページの3の続き）

次に述べるようにこの国際出願に二以上の発明があるところこの国際調査機関は認めた。

（発明群1）請求項1，2

特定構造を有する細胞品質評価装置、及び、当該装置を用いた細胞品質評価方法 に係る発明。

（発明群2）請求項3－15，16－20

心筋拍動のフィールド・ポテンシャル波形のゆらぎの大きさを、隣接する拍動信号の比較によって計測することを特徴とする、心筋毒性検査装置、及び、心筋毒性検査方法 に係る発明。

なお、請求項14，15は、発明群1に属する請求項1，2も引用しているが、発明群1には「心筋毒性検査装置」は記載されていないから、当該引用部分は除外するものとする。

1. ☐ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求項について作成した。
2. ☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求項について調査することができたので、追加調査手数料の納付を求めなかった。
3. ☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求項のみについて作成した。
4. ☒ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求項について作成した。

請求項1－2

追加調査手数料の異議の申立てに関する注意

- ☐ 追加調査手数料及び、該当する場合には、異議申立手数料の納付と共に、出願人から異議申立てがあった。
- ☐ 追加調査手数料の納付と共に出願人から異議申立てがあったが、異議申立手数料が納付命令書に示した期間内に支払われなかった。
- ☐ 追加調査手数料の納付はあったが、異議申立てはなかった。



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(54) Title: SYSTEM AND METHOD FOR MONITORING CARDIOMYOCYTE BEATING, VIABILITY, MORPHOLOGY AND
ELECTROPHYSIOLOGICAL PROPERTIES

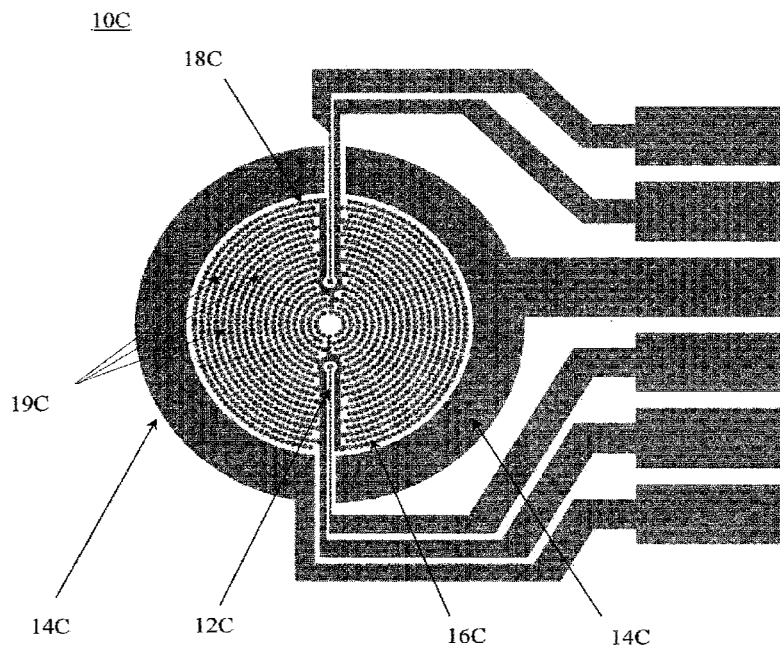


FIG. 2C

(57) Abstract: Devices, systems and methods
for monitoring excitable cells, such as cardi-
omyocytes, on microelectrode arrays that
couple the electro-stimulation of excitable
cells to induce or regulate cardiomyocyte
beating and the simultaneous measurement of
impedance and extracellular recording to as-
sess changes in cardiomyocyte beating, viab-
ility, morphology or electrophysical proper-
ties in response to a plurality of treatments.



UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *the filing date of the international application is within two months from the ~~date~~ of expiration of the priority period (Rule 26bis.3)*

SYSTEM AND METHOD FOR MONITORING CARDIOMYOCYTE BEATING, VIABILITY, MORPHOLOGY AND ELECTROPHYSIOLOGICAL PROPERTIES

5 TECHNICAL FIELD

This invention relates to the field of cell-based assays and more specifically to devices, systems and methods for electro-stimulation of excitable cells, such as cardiomyocyte or cardiomyocyte precursor cells and performing extracellular recording
10 and/or impedance monitoring of electro-stimulated cells.

BACKGROUND OF THE INVENTION

15 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 or cell-substrate impedance is measured and analyzed to monitor cellular changes, such as
20 changes in cell morphology. For example, PCT Application No. PCT/US03/22557, titled “Impedance Based Devices and Methods for Use in Assays”, discloses a device for detecting cells and/or molecules on an electrode surface. 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
25 the device are disclosed, together with the apparatuses, and systems for using such devices to perform certain cell based assays.

Bringing a new drug to the market can take anywhere between 8 to 16 years, and the average cost of developing a drug is now around \$500 - \$800 million with the cost expected to hit the \$1 billion mark within the next four years. Cardiotoxicity has been
30 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.

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

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 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 format, which even though is available in last several years is not yet widespread. Another 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 to utilize the differentiation of embryonic stem (ES) cells into 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 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 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 can serve as an excellent 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 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 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 techniques and therefore an enriched cardiomyocyte population can be obtained.

Technologies designed to assess cardiomyocyte behavior 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. Systems such as automatic patch clamp instrumentation that are available can monitor a single cardiomyocyte at a time and with very limited throughput.

In US 2011/0039294 an approach to monitoring a cardiomyocyte population is disclosed, which includes both impedance monitoring and extracellular recording technologies with high precision. While acceptance of the technology is increasing, there still remain challenges when working with the cells themselves. For example, when using primary cardiomyocyte cells harvested from tissue or after extended culturing, the beating of cells can stop or slow. Accordingly, there is a need to provide devices, systems and methods that assess the cardiotoxicity of compounds while working to ensure the continued or regular beating of cardiomyocyte cells.

SUMMARY OF THE INVENTION

The present invention discloses devices, systems and methods for performing impedance monitoring and/or extracellular recording of excitable cells, such as cardiomyocytes in cell based assays that encourage the continued and regular beating of

excitable cells such that the cardiotoxicity of potential drugs can be assessed. In addition, the devices, systems and methods provide improved characterization of excitable cells in response to compound administration to assess responses of cardiomyocytes including cardiotoxic effects, while increasing efficiency of throughput for potential drug candidates. The invention further provides improved characterization of cells during differentiation processes related to development of cardiomyocytes.

In one aspect of the invention a system for monitoring excitable cells is disclosed, which includes a device having at least one well, each well having a bottom having a nonconductive substrate, wherein the substrate has a surface suitable for attachment of excitable cells; a power source configured to deliver an electrical signal capable of electro-stimulating excitable cells; and at least one analyzing module for measuring an electrical property from electro-stimulated excitable cells, characterized in that each well includes a pair of electro-stimulation electrodes configured to receive the electrical signal from the power source thereby delivering an electro-stimulating signal to the well for electro-stimulation of excitable cells attached to the substrate; and at least a second pair of electrodes communicatively coupled to the at least one analyzing module, which is selected from the group consisting of a pair of impedance monitoring electrodes communicatively coupled to the at least one analyzing module in the form of an impedance analyzer thereby permitting impedance monitoring of excitable cells attached to the substrate, and an extracellular recording electrode pair communicatively coupled to the at least one analyzing module in the form of an extracellular recording amplifier thereby permitting extracellular recording of excitable cells attached to the substrate.

In regards to electro-stimulation, in some embodiments the electrical signal is a series of pulses at a regular time interval, such as but not limited to 0.5 seconds to 2 seconds. The electrical signal can vary but is preferably 1V to 2.5V for 0.5-2 milliseconds. A percentage of a surface area of the bottom of the at least one well occupied by the pair of electro-stimulation electrodes can vary but in some embodiments is selected from the group consisting of 5% or more, 10% or more, 20% or more, 30% or more, 50% or more, and 70% or more. The electro-stimulation electrodes independently includes an unbranched electrode structure or a branched electrode structure.

In preferred impedance monitoring configurations, the at least second pair of electrodes is the pair of impedance monitoring electrodes and the analyzing module is in the form of the impedance analyzer. The pair of impedance monitoring electrodes can be provided in a variety of configurations but is preferably a pair of interdigitated electrode structures, wherein each electrode structure comprises a plurality of electrode elements. In addition, the pair of impedance monitoring electrodes can be a pair of electrode structures having a same surface area. In preferred embodiments the impedance analyzer monitors impedance at millisecond time resolution. In further embodiments, the extracellular recording electrode pair as a third pair of electrodes. In still further embodiments at least one electrode of the pair of electro-stimulation electrodes is also at least one electrode of the extracellular recording electrode pair thereby permitting electro-stimulation of excitable cells and extracellular recording of attached cells using a same electrode at different time points.

In regards to extracellular recording, the at least second pair of electrodes can be the extracellular recording electrode pair and the analyzing module can be the extracellular recording amplifier. Preferably, the extracellular recording electrode pair includes a recording electrode and a reference electrode. In some embodiments the recording electrode has a diameter from about 10 μm to about 200 μm or from about 30 μm to about 100 μm . In some embodiments, the extracellular recording electrode pair comprises a recording electrode and a reference electrode, further wherein a ratio of the area of reference electrode to the area of recording electrode is selected from the group consisting of 2 or more, 10 or more, 100 or more, 1,000 or more, and 10,000 or more. In further embodiments a second recording electrode is provided having a same diameter as a first.

In some extracellular recording embodiments, at least one electrode of the pair of electro-stimulation electrodes is also at least one electrode of a pair of impedance monitoring electrodes thereby permitting electro-stimulation of excitable cells and impedance monitoring of attached cells using a same electrode at different time points and thus electro-stimulation together with simultaneous impedance monitoring using as few as four total electrodes. In such embodiments, preferably the pair of impedance monitoring electrodes is a pair of interdigitated electrode structures, wherein each

electrode structure comprises a plurality of electrode elements. In variations of the extracellular recording embodiments, at least one electrode of the pair of impedance monitoring electrodes is the reference electrode.

In addition a device for monitoring excitable cells is also provided, the device including at least one well, each well having a bottom having a nonconductive substrate, wherein the substrate has a surface suitable for attachment of excitable cells; a pair of electro-stimulation electrodes positioned on the substrate within the at least one well and configured to electro-stimulate the excitable cells; and at least a second pair of electrodes positioned within the at least one well and selected from the group consisting of a pair of impedance monitoring electrodes and extracellular recording electrode pair, wherein the pair of impedance monitoring electrodes is configured for monitoring cell-substrate impedance of cells attached to the substrate, and wherein the extracellular recording electrode pair is configured for monitoring extracellular potential of cells attached to the substrate.

In regards to electro-stimulation electrodes a percentage of a surface area of the bottom of the at least one well occupied by the pair of electro-stimulation electrodes can vary but may be selected from the group consisting of 5% or more, 10% or more, 20% or more, 30% or more, 50% or more, and 70% or more. In some embodiments each of the electro-stimulation electrodes independently include an unbranched electrode structure or a branched electrode structure.

In some embodiments, the at least second pair of electrodes is the pair of impedance measurement electrodes. In such configurations each electrode within the pair of impedance measurement electrodes can include a plurality of electrode elements and can be configured as one half of a pair of interdigitated electrodes. In some embodiments, electrode of the pair of impedance monitoring electrodes has a same surface area.

In regards to extracellular recording configurations, the at least second pair of electrodes can be the extracellular recording electrode pair. Preferably, the extracellular recording electrode pair includes a recording electrode and reference electrode; however, two or more recording electrodes may be associated with one or more reference electrodes. Preferably each recording electrode has a diameter from about 10 μm to

about 200 μm or from about 30 μm to about 100 μm . The ratio of the area of reference electrode to the area of recording electrode can vary but may be selected from the group consisting of 2 or more, 10 or more, 100 or more, 1,000 or more, and 10,000 or more. The reference electrode of the recording and reference electrode pair can have a branched or an unbranched structure.

In some embodiments at least one electrode is shared between the pair of electro-stimulation electrodes and the at least second electrode pair. In such configurations, the at least second electrode pair is the pair of impedance measurement electrodes. In other configurations the at least second electrode pair is the extracellular recording electrode pair.

In still further embodiments of the device the at least second electrode pair is the pair of impedance measurement electrodes, and device further includes the extracellular recording pair as a third pair of electrodes. In such configurations, at least one electrode can be shared between the pair of impedance measurement electrodes and the recording and reference electrode pair.

In another aspect of the invention a method for monitoring excitable cells is provided, which includes providing a system for electro-stimulating excitable cells and monitor impedance and/or extracellular potential of stimulated cells which has at least two pairs of electrodes; adding a sample of excitable cells to the device; electro-stimulating the excitable cells with electro-stimulation electrodes; and monitoring electro-stimulated cells through the at least second pair of electrodes. In some embodiments the excitable cells are cardiomyocytes or cardiomyocyte precursor cells. Electro-stimulation can be performed at a plurality of time intervals, optionally at regular time intervals.

When impedance monitoring, a pair of impedance monitoring electrodes can communicatively coupled to an impedance analyzer, thereby permitting the step of monitoring electro-simulated cells to include monitoring impedance of electro-stimulated cells. Preferably, impedance is monitored in millisecond time resolution. The methods may also include adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well for analysis. The methods may include calculating and comparing an impedance-based parameter prior to and after

adding the compound to identify whether a change occurs in the excitable cells in response to the compound. In some embodiments, the impedance-based parameter is compared between at least two different electro-stimulation intervals. Examples of suitable impedance-based parameter include an impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated from the cell index. Preferably, the impedance based parameter is plotted as an impedance-based curve over time and the step of comparing the impedance-based parameter between at least two different electro-stimulation intervals includes comparing impedance-based curves between at least to different electro-stimulation intervals. In some embodiments, the excitable cells are added to each of at least two wells and the step of performing impedance measurements is performed for each of the at least two wells, the method can therefore further include adding a compound suspected of affecting excitation contraction coupling of the excitable cells to a first of the at least two wells to form a test well and adding a control to a second of the lease two wells to form a control well, and comparing an impedance-based parameter between the test well and control well. Again, suitable impedance-based parameters can be the impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated from the cell index. Preferably, the impedance-based parameter is plotted as an impedance-based curve over time and the step of comparing the impedance-based parameter between the test well and control well includes comparing the impedance-based curve of the test well to the impedance based curve of the control well.

In some embodiments, the extracellular recording electrode pair is communicatively coupled to the an extracellular recording amplifier, and the step of monitoring electro-simulated cells includes extracellular recording of electro-stimulated cells. Preferably, the embodiments include plotting extracellular potential from the extracellular recording of electro-stimulated cells over time to form a field potential curve. Extracellular recording can be performed before and after the step of electro-stimulating the excitable cells.

In further embodiments, the method includes adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well for extracellular recording. In such embodiments, extracellular potential of the excitable

cells prior to and after adding the compound can be compared to identify changes in extracellular potential in response to the compound. Extracellular potential may be plotted over time to form a field potential curve for analysis.

In further embodiments, excitable cells are added to each of at least two wells and the step of performing extracellular recording measurements is performed for each of the at least two wells. In such instances, the method can include adding a compound suspected of affecting excitation contraction coupling of the excitable cells to a first of the at least two wells to form a test well and adding a control to a second of the at least two wells to form a control well, and comparing extracellular potential between the test well and control well. Extracellular recording can be performed before and after adding the compound.

In some embodiments, a pair of impedance monitoring electrodes are communicatively coupled to the impedance analyzer and the extracellular recording electrode pair is communicatively coupled to the extracellular recording amplifier, and the step of monitoring electro-stimulated cells includes impedance monitoring and extracellular recording of electro-stimulated cells. In some embodiments at least one electrode is shared between two pairs of electrodes selected from the group consisting of the pair of electro-stimulation electrodes and the pair of impedance monitoring electrodes, the pair of electro-stimulation electrodes and the extracellular recording electrode pair, and the pair of impedance monitoring electrodes and the extracellular recording electrode pair. In such configurations, the sharing of the electrode(s) is performed by switching communication to the at least one electrode.

In some embodiments the method include adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well. Such methods may also include comparing an impedance-based parameter and extracellular field potential prior to and after adding the compound to identify whether a change occurs in the excitable cells in response to the compound. Preferably, the impedance-based parameter is selected from the group consisting of an impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated from the cell index. In still further embodiments, the impedance based parameter is plotted as an impedance-based curve over time and the extracellular field

potential is plotted as field potential over time and the step of comparing the impedance-based parameter and extracellular field potential between at least two different electro-stimulation intervals includes comparing impedance-based curves and comparing field potential curves between at least two different electro-stimulation intervals.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration showing a correlation of a cell index impedance parameter and field potential for a contraction-relaxation interval in a primary neonatal rat cardiomyocyte.

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FIGS. 2A-D depict different configurations of electrode arrays **10A-D** that can perform electro-stimulation, impedance measurement and extracellular recording.

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FIG. 3 depicts an exemplary system of a device **100**, a combined analyzer **200** having switchable electro-stimulation, impedance monitoring, and extracellular recording functions and computer **300**.

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FIGS. 4A-D depict examples of the graphical interface for software used in data acquisition (**FIGS 4A-C**) and data analysis (**FIG. 4D**).

FIG. 5 shows an exemplary cardio experimental work flow chart.

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FIGS. 6A-B show results from extracellular recording of field potential and impedance monitoring of HL-1 cells (**FIG. 6A**) and CorAT cells (**FIG. 6B**).

FIGS 7A-C show results from extracellular recording of field potential and impedance monitoring of CorAT cells with the addition of Solatol at 0 μM (**FIG. 7A**), 200 μM (**FIG. 7B**) or 400 μM (**FIG. 7C**).

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FIGS. 8A-F show results from extracellular recording of field potential and impedance monitoring of rat primary cardiomyocyte cells with the addition of Quinidine at 0 μ M (**FIG. 8A**), 2 μ M (**FIG. 8B**), 4 μ M (**FIG. 8C**), 8 μ M (**FIG. 8D**) or 16 μ M (**FIG. 8E**). **FIG. 8F** shows an overlay of an interval of ECR field potentials from each of **FIGS. 8A-E**. **FIG. 8G** shows an overlay of an interval of impedance from each of **FIGS. 8A-E**.

FIG. 9 shows changes in impedance and extracellular recording of field potential of Cor.AT cells during electrostimulation and without electrostimulation.

FIG. 10 shows changes in impedance and extracellular recording of field potential of reat primary cardiomyocyte during electrostimulation and without electrostimulation.

FIGS. 11A-F shows a series of curves from an experiment where HL-1 cells (**FIG. 11A**), were electro-stimulated to induce measurable changes in field potential (FP) (**FIG. 11B**), which could then be modulated through the addition of different concentrations of the calcium channel blocker Israpidine (**FIGS. 11C-E**). **FIG. 11F** being an overlay of field potential curves from **FIGS. 11A-E**.

FIGS. 12A-G show a series of curves from an experiment where HL-1 cells (**FIG. 12A**) where electrostimulated to induce measureable changes in field potential (FP)(**FIG. 12B**). A calcium channel activator was added to culture and modulation of the field potential (FP) was detected (**FIG. 12C**). The culture was paced use electro-stimulation (**FIG. 12D**). Addition of different concentrations of antagonist (**FIGS. 12E-F**) were added and modulated the field potential (FP). **FIG. 12G** is an overlay of **FIGS. 12A-F**)

FIGS. 13A-C show a series of curves from an experiment documenting the electro-stimulation of CorAT cells and the pacing of cardiomyocytes where **FIG. 13A** overlays impedance (via cell index) and field potential in response to electro-stimulation

and after electro-stimulation stops. In **FIG. 13B**, impedance and field potential are split in that the upper panel is impedance and the lower panel is field potential. **FIG. 13C** is an enlarged view showing a single interval and the relationship between impedance and field potential.

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FIGS. 14A-B show the electro-stimulation of rat primary cardiomyocytes and the impedance and field potential profiles in response to the regular pacing of cardiomyocytes.

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FIGS. 15A-B show results from experiments monitoring the field potential of neurons after treatment with bicuculline (**FIG. 15A**) or Glutamate (**FIG. 15B**).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

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As an introduction, we describe label-free methods for electro-stimulating and monitoring excitable cells, such as cardiomyocytes, *in vitro*. It is an object of the invention to apply appropriate electro-stimulation signals for stimulating and/or pacing excitable cells, together with extracellular recording of cells, impedance monitoring of cells, or parallel impedance monitoring and extracellular recording cells using microelectrodes to non-invasively monitor cells. To this end, the devices, systems and methods allow the continuous monitoring of cardiomyocyte viability overtime and can monitor the interaction of compounds which ultimately result in promoting loss of cardiomyocyte viability. Further, the devices systems and methods permit high resolution monitoring of the beating cycle of a population of cardiomyocyte cells thus can detect changes in cell beating, such as shifts in contraction or relaxation that would be difficult to detect otherwise. As further introduction, an exemplary plot of the relationship between impedance and extracellular recording is shown in **FIG. 1**, where high resolution monitoring demonstrates that contraction and relaxation of cells, such as cardiomyocytes can be effectively monitored using an impedance-based approach and more preferably together with extracellular recording. In particular a peak in impedance

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(as measured in cell index) is followed by a peak in field potential. According changes in either impedance or extracellular recording provides insight as to changes occurring in the excitable cell population. Still further, by incorporating short term impedance monitoring with long term impedance monitoring the status excitable cell population can be further studied, An implication of this high resolution monitoring discussed herein is that impedance monitoring and extracellular recording can be used as a high throughput approach to screen for potential cardiotoxic affects of compounds. Further it also provides approaches for monitoring embryonic stem cell development into cardiomyocytes and implications associated with genetic knock outs and transgene expression at different developmental stages.

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 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 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.

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

As used herein, “biomolecular coating” , “biological molecule coating” or “coated with a biomolecule” refers a coating on a surface 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, 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.

5 An “organic compound coating” or a “coating having an organic compound”) as used herein refers to a coating on a substrate 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.

10 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
15 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”, “impedance monitoring electrode” , “impedance measurement electrode” or “impedance electrode structure” is a structure, such as an electrode, used for impedance monitoring. An
20 “impedance electrode” may also operate as an extracellular recording electrode or electro-stimulation and thus may provide both impedance monitoring and extracellular recording measurements or both impedance monitoring and electro-stimulation, albeit at different time points.

25 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.

30 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.

5 As used herein, a “pair of electrodes” or “electrode pair” 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
10 surface. Non-limiting examples of electrode structure units are interdigitated electrode structure units and concentric electrode structure units.

As used herein “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
15 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.

As used herein “electrode traces” or “electrically conductive traces” or “electrical traces”, are electrically conductive paths that extend from electrodes or electrode
20 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 a electro-stimulation power source, an analyzer or amplifier, such as an impedance analyzer or amplifier, a voltage amplifier and the like. Electrical communication of electro-stimulation electrodes, impedance electrodes or extracellular recording electrodes
25 typically involves connection to a connection pad using an “electrode trace.”

As used herein “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
30 circuit or a signal source or an extracellular voltage signal amplifier). The electrical 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.

5 As used herein “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).

10 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.

15 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.

20 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.

25 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
30 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 preferably, the at least two electrodes of an electrode structure have nearly identical or identical surface area.

5 As used herein, “the device has a surface suitable for cell attachment or growth” 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
10 thereof, contain substances necessary for cell viability or growth. These 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
15 surface within twelve hours. More preferably, a surface that is 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
20 within twelve hours of plating. Most 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 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
25 structures”) 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 surface,
30 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 waveform) 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, “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.

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 well contains an identical number of cells.

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 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.

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.

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 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 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 (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 long as days or weeks.

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 an ion channel. In still another application, a compound is capable of, or is suspected of, modulating cardiomyocyte excitation contraction coupling or beating. 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.

As used herein "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.

5 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
10 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.

15 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 derive of the Cell Index with respect to time, divided by the Cell Index at the time point. In other words, CCI is calculated as

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

20 As used herein “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
25 inside a cell through the cell membrane, the extracellular recording electrodes are located outside of the cells.

Devices and Systems for Electro-Stimulation and Measurement of Excitable Cells

Provided herein are devices and systems that permit the electro-stimulation as well as extracellular recording and/or impedance monitoring of cells. In particular, an exemplary device includes at least one well, each well having a bottom having a nonconductive substrate, the substrate having a surface suitable for attachment of excitable cells; a pair of electro-stimulation electrodes positioned on the substrate within the at least one well and configured to electro-stimulate the excitable cells; and at least a second pair of electrodes positioned within the at least one well. The at least second pair of electrodes can be a pair of impedance monitoring electrodes or an extracellular recording electrode pair. In some embodiments, the second pair of electrodes is the pair of impedance monitoring electrodes and the extracellular recording pair is provided as a third electrode pair. The pair of impedance monitoring electrodes are configured to measure cell-substrate impedance of cells attached to the substrate, and the extracellular recording electrode pair are configured to measure extracellular potential of cells attached to the substrate. Accordingly, the device permits the electro-stimulation of excitable cells, such as cardiomyocytes while various embodiments permit impedance monitoring, extracellular recording or both impedance monitoring and extracellular recording of excitable cells.

The above is accomplished at least in part because while the substrate has a surface suitable for attachment of excitable cells, it has been found that the attachment of excitable cells on the substrate provides a suitable form for measuring extracellular potential through the extracellular recording electrode pair, and measuring cell-substrate impedance through the pair of impedance monitoring electrodes, and still further that the attached excitable cells can be electro-stimulated or paced by electro-stimulation when appropriate electrical signals are delivered to the pair of electro-stimulation electrodes. Thus, by providing a device that permits alternating electro-stimulation coupled with cell detection or measurement techniques the cell culture can maintain a regular beating interval to test the cardiotoxic affects of various compounds or monitor the development of cardiomyocyte precursor cells to cardiomyocytes.

Preferably, the device includes one or more fluid-impermeable receptacles which serve as fluid containers or wells. Such receptacles may be reversibly or irreversibly attached to or formed within the substrate or portions thereof (such as, for example, wells

formed as in a microtiter plate). 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 some embodiments, commercial tissue culture plates can be adapted to fit the substrate. Bottomless plates may also be custom-made to preferred dimensions. The device may have any number of wells as desired for the particular experiment. For instance, the device may have 1 well, 2 wells, 3 wells, 6 wells, 8 wells, 12 wells, 24 wells, 36 wells, 96 wells, 384 wells, 1536 wells or the like. 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.

The surface of the substrate is suitable for cell attachment and optionally growth. Preferably, the nonconducting substrate is planar, and is flat or approximately flat. The substrate 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 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 applying a biocompatible or biomolecular coating with a suitable material, such as a biocompatible polymer or the like. Further, attachment or growth along the substrate or electrodes may be enhanced by pre-coating the substrate with a protein or compound that facilitates 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 or binding moieties such as adhesion molecules that enhance cell attachment, particularly ES cells that are cardiomyocyte precursor cells or cardiomyocytes.

5 To improve efficiency of production, electrodes of the invention may be applied to the substrate followed by joining the electrode applied substrate to a plate of bottomless wells or may be applied to a well already having the substrate as a bottom. Electrodes may be formed from larger sheets of conductive metal, such as via laser ablation of a metallic film and may be applied directly to the substrate. Alternatively,
10 electrodes may be printed on the substrate using printing techniques such as those similar to ink-jet printing where a conductive fluid having ultraviolet (UV) curable monomers, polymers or compounds is printed on the substrate, then a light source is applied to cure the applied conductive fluid to form electrodes. The skilled artisan will appreciate that conductive material may be applied directly to a planar substrate or may be inserted into
15 grooves laser ablated or formed into the substrate surface. A glue, such as a UV curable glue, can be applied between the substrate and electrode or above the electrode for added security. Further, when applying conductive fluids, it may be preferred to apply a mask prior to applying the fluid to further define the electrode area.

Examples of suitable electrode configurations are shown in **FIGS. 2A-2D**, which
20 depict examples where electrodes are shared between different functional pairs, in particular, where two electrodes from the pair of electro-stimulation electrodes are shared with a pair of impedance monitoring electrodes. Using this technical approach the pair of impedance monitoring electrodes can be used for electro-stimulating the excitable cells when not measuring impedance. Thus, the electrode arrays provide electrode structures
25 for cell electro-stimulation, cell-impedance measurement and extra-cellular recording, as designed on a non-conductive substrate associated with a single well. Turning in more detail to **FIG. 2A** an exemplary electrode array **10A** includes an extracellular recording electrode pair formed from two round-circular extra-cellular recording electrodes **12A** positioned at about the middle of the entire array **10A** and a unitary one-piece reference
30 electrode **14A** positioned at opposing ends of the array **10A**. A pair of interdigitated electrodes (**16A**, **18A**) formed from a plurality of electrode elements **19A** function to

electro-stimulate a cell population when switched to a electro-stimulation mode and function to measure cell substrate impedance when switched to an impedance monitoring mode. These shared electro-stimulation/impedance monitoring electrodes **16A**, **18A** are shown in a branched configuration. More specifically, a plurality of electrode elements **19A** of each electrode structure **16A**, **18A** are shown having a circle on line configuration.

As a nonlimiting example, particular specifications shown in **FIG. 2A** are that the diameter of each of the two the recording electrodes **12A** is 100 μm ; the distance between two recording electrodes **12A** is 2.98 mm; the diameter of circles in the circle-on-line electrode elements **19A** is 90 μm , the center-to-center distance between two adjacent circle-on-line electrode elements **19A** is 110 μm ; and the gap between two shared impedance/electro-stimulation electrodes **16A**, **18A** and covering the recording electrodes **12A** is $\sim 290 \mu\text{m}$. Each of the electrodes **12A**, **14A**, **16A**, **18A** are connected to a connection pad **26A** via an electrical trace **26A**

A variety of other configurations have been developed including electrode structures for cell electro-stimulation, cell-impedance measurement and extracellular recording of cells, as designed on a non-conductive substrate associated with a single well. For example, in **FIG. 2B**, each electrode array **10B** includes two round-circular extracellular recording electrodes **12B** and a single unitary one-piece reference electrode **14B**. Electrode structures **16B**, **18B** are used for both electro-stimulation and cell impedance monitoring albeit at different time points by electronic switching. In this exemplary embodiment, the diameter of each recording electrode **12B** is 60 μm ; the distance between two recording electrodes **12B** is 2 mm; the diameter of circles in the circle-on-line electrode elements **19B** is 90 μm , the center-to-center distance between two adjacent circle-on-line electrode elements **19B** is 110 μm ; the gap between two impedance-electrode-structures **16B**, **18B** and covering the recording electrodes **12B** is $\sim 290 \mu\text{m}$.

Still another configuration is shown in **FIG. 2C**, where each electrode array **10C** is configured on a non-conductive substrate associated with a single well. The electrode array **10C** includes a round-circular extra-cellular recording electrode **12C** and a unitary one-piece reference electrode **14C**. Electrode structures **16C**, **18C** perform both

impedance measurement and electro-stimulation of cells at different time points by electronic switching. In this example, a plurality of circle-on-circular-line electrode elements **19C** form interdigitated electrode structures **16C**, **18C**. In this exemplary embodiment, the diameter of the recording electrodes **12C** is 80 μm ; the distance between two recording electrodes **12C** is 1.44 mm; the diameter of circles in the circle-on-line electrode elements **19C** is 90 μm , the center-to-center distance between two adjacent circle-on-line electrode elements **19C** is 110 μm .

Still another configuration is shown in **FIG. 2D**, where an electrode array **10D** includes two round-circular extra-cellular recording electrodes **12D** and a single unitary one-piece reference electrode **14D**. Electrode structures **16D**, **18D** are used for cell impedance monitoring. Electrode Structures **20D**, **22D** are used for electro-stimulation and for cell impedance monitoring. In this exemplary embodiment, the diameter of each recording electrode **12D** is 60 μm ; the distance between two recording electrodes **12D** is 2 mm; the diameter of circles in the circle-on-line electrode elements **19D** is 90 μm , the center-to-center distance between two adjacent circle-on-line electrode elements **19D** is 110 μm ; the gap between two impedance-electrode-structures **16D**, **22D** and covering the recording electrodes **12D** is $\sim 290 \mu\text{m}$.

In regards to the device generally, configurations including two or more wells, preferably electrodes within each well of the device are individually addressed, meaning that electrical traces and connection pads of the arrays are configured such that an array can be connected to its power source, impedance amplifier or extracellular recording amplifier independent of the operation of an array in a neighboring well and thus each well can operate using different electro-stimulation intervals, perform different measurements and the like without adversely affecting neighboring wells.

Electrical traces of conductive material used to connect each of the electrodes to a corresponding connection pad 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.

Turning to the pair of electro-stimulation electrodes generally, the electrodes may be provided in a variety of shapes and configurations so long as the configuration permits

electro-stimulation. Electro-stimulation can be accomplished using a variety of different waveforms such as rectangular, ramp, sinusoidal signals and the like. Either uni-polarity or bi-polarity signals can be used, with signal amplitude ranging from -2.5 V to +2.5 V. When pacing cardiomyocytes electrical signal from between 1V-2V was preferred while
5 electrical signal between about 1.1-1.3V was most preferred. In preferred embodiments, the electro-stimulation voltage can be controlled at voltage resolutions up to 2 mV. Maximum frequency of the stimulation signals could be up to 50 kHz.

Preferably, each of the pair of electro-stimulation electrodes of an electrode array is connected to a separate connection pad, which is preferably located at the edge of the
10 substrate. Connecting the pair of electro-stimulation electrodes to the connection pads can be performed by applying electrical traces of conductive material therebetween. This facilitates connection to a suitable power source by providing an interface at which the power source can connect. Connection to the connection pads is generally performed through the use of electrically conductive pins, clips or the like.

15 One or more electro-stimulation electrodes can be shared with either the extracellular recording electrode pair or the pair of impedance monitoring electrodes by timing the switching between the power source that delivers the electro-stimulation signal through the electro-stimulation electrodes and the extracellular recording amplifier or the impedance analyzer.

20 Turning now to the extracellular recording electrode pair generally, extracellular recording is conducted by amplifying and recording electrical voltage signals between a recording electrode(s) and reference electrode(s). 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
25 of different ion channels across cell membrane during the action potential duration. 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 voltage signals and to ensure that the majority, if not all, of the recording voltage signals are derived from that on the recording electrode.
30 Thus, 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. For example, **FIG. 2A** shows a schematic representation of such electrode pairs placed on a non-conductive substrate, including a small area recording electrode **12A** and a much larger area reference electrode **14A**. As shown throughout **FIGS. 2A-2D**, preferably the reference electrode **14** is positioned towards the perimeter of the electrode array **10**; however, it must not physically contact the recording electrode **12**.

The reference electrode generally, can be 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 reference electrode has a branched configuration, which may result in a large surface for the reference electrode. In some embodiments, the ratio of the surface area of the reference electrode to that of the recording electrode is more than 2. In other embodiments, the ratio of the surface area of the reference electrode to that of the recording electrode is 10 or more than 10. In still other embodiments, the ratio of the surface area of the reference electrode to that of the recording electrode is 100 or more than 100. In other embodiments the ratio of the surface area of the reference electrode to that of the recording electrode is 1000 or more than 1000. In other the ratio of the surface area of the reference electrode to that of the recording electrode is 10,000 or more than 10,000.

While it is preferable to simultaneously measure impedance and perform extracellular recording, in some embodiments one or both electrodes of the pair of impedance measurement electrodes is shared with the extracellular recording electrode pair. When using impedance electrodes in the form of interdigitated electrode structures having a plurality of electrode elements, typically the shared electrode would be used as a reference electrode in the extracellular recording electrode pair. This can be accomplished when the surface area of the impedance monitoring electrode(s) is sufficiently larger than the surface area of the recording electrode to act as a reference electrode. The skilled artisan will appreciate that by electrically switching a pair of interdigitated impedance electrodes from impedance monitoring to function as a single reference electrode, the surface area ratio of the combined interdigitated electrodes to

recording electrode would substantially increase and thus may be preferable in some instances. Further, it is also possible, though not preferred to utilize an impedance measurement electrode as a recording electrode when the reference electrode is sufficiently larger than the impedance measurement electrode. While not preferred this approach is more likely when using impedance measurement electrode configurations having a small working electrode and large counter electrode as previously detailed in the art.

Turning now to the pair of impedance monitoring electrodes themselves, each pair includes two or more electrode structures that are constructed to have dimensions and spacing such that they can, when connected to an impedance analyzer, operate as a unit to generate an electrical field in the region of spaces around the impedance electrode structures. Preferably the electric field is substantially uniform across the pair of impedance electrodes. In preferred embodiments, the pair of impedance electrodes includes two impedance measurement electrode structures, each of which includes multiple electrode elements, or substructures, which branch from the electrode structure. In preferred embodiments, the electrode structures in each pair have substantially the same surface area.

Each of the two complementary impedance monitoring electrode structures of a pair connect to a separate connection pad that is preferably located at the edge of the substrate. In some embodiments, the array includes two pairs of impedance monitoring electrodes separated by a recording electrode region where one or two recording electrodes are positioned on a same plane of the substrate. In such embodiments it is preferred that each electrode structure be assigned to a separate connecting pad however the pairs could be electrically joined such as by connection at a shared connection pad or through an electronic switch.

In some embodiments, for each of two or more pairs of impedance monitoring electrodes in two or more wells, a first of the two impedance monitoring electrode structures is connected to one of two or more connection pads, and the second of the two impedance monitoring electrode structures is connected to another of the two or more connection pads.

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

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

Achieving an approximately uniform distribution across the pair of impedance measurement electrodes 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 pair is approximately equal to the resistance at any single other location on the pair. In most embodiments, the electrode elements (or electrode structures) of a given pair will have even spacing and be of similar thicknesses and widths, the electrode buses of a given pair will be of similar thicknesses and widths, and the electrode traces leading from a given pair to a connection pad will be of closely similar thicknesses and widths. Thus, in these preferred embodiments, a pair of electrode structures 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 pair.

In some preferred embodiments of impedance measurement configurations, 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 pair, for any two locations on the pair 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 pair to provide for uniform resistances across the array.

When incorporating configurations having two or more wells, impedance measurement electrodes between electrode arrays of different wells can share a connection pad. Preferably one of the impedance measurement electrode structures of at least one of the electrode arrays of the device is connected to a connection pad that also connects to an impedance measurement electrode structure of at least one other of the

electrode arrays of another well device. Preferably for at least two arrays of the device, each of the two or more arrays has a first impedance monitoring electrode structure connected to a connection pad that connects with an impedance measurement electrode structure of at least one other electrode array of another well, and each of the two or more
5 arrays has a second impedance monitoring 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 configurations of a device there are at least two electrode arrays each of which has a first impedance monitoring electrode structure that is connected to a common connection pad and a second impedance monitoring electrode
10 structure that is connected to an independent connection pad.

Preferred arrays for devices of the present invention include at least one pair of impedance measurement electrodes, each comprising two electrode structures, such as, in the form of a spiral configuration or an interdigitated configuration. Preferably the pair of impedance monitoring electrodes are fabricated on the substrate, in which the pair comprises two impedance monitoring electrode structures, each of which comprises multiple circle-on-line electrode elements, in which the electrode elements of one electrode structure alternate with the electrode elements of the opposite electrode structure. The pair of impedance monitoring electrodes may be provided in configurations, such as interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal and castellated.

A central advantage to systems described herein is that they can incorporate two separate and complete recording systems into one single instrument, namely, an extracellular signal recording system and impedance measurement system. This is accomplished by collecting field potential, which is a local readout such as by an
15 extracellular recording electrode and impedance monitoring, which is an entire well readout by at least one pair of impedance electrodes respectively. In addition, with the capability of simultaneous recording of extracellular recording and impedance, the interaction of the results obtained from these separate recording system can be used to support each other.

20 Parallel impedance-based monitoring as well as extra-cellular recording based monitoring of cardiomyocytes, under the condition of electro-stimulation 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 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 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. Also, the systems of the present invention can also record cardiomyocyte action potentials or field potentials, allowing detailed electrophysiological analysis and assessment of cardiomyocytes for relatively short duration at a time. Furthermore, the ability of electro-stimulating cardiomyocytes allow for electrophysiological measurement and impedance measurement of otherwise non-beating cardiomyocytes. The synchronization of electro-stimulation with extra-cellular potential recording and cell impedance measurement would allow better and precise control of initiation timing points for excitation process and allow more reproducible measurement of extra-cellular potential and cell impedances.

In view of the above with or without applying electro-stimulation signals to stimulate or pace cardiomyocytes, 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 cardiac culture preparation, impedance monitoring reflects the cell growth, which can be correlated through an impedance-based curve, namely, a cell index curve

and contraction / relaxation function by impedance beating signals; while extracellular recording reflects cardiac excitation and conduction by field potential signals. Further, this the invention provides the ability to 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.

While the invention is primarily discussed in regards to cardiotoxicity and monitoring cardiomyocytes, the devices and systems can also be used to study a neuronal culture preparation, in which impedance monitoring reflects the cell growth, which can be correlated through a cell index growth curve. In addition as with electro-stimulation of cardiomyocytes, an impedance electrode can also serve for stimulation in the neuronal cell preparation, while evoking a response for network stimulation by either chemical or electrical could be recorded at the extracellular recording electrode(s). Theoretically, the configurations could also be used for any excitable cell cultures and tissue slices, so the system possesses high capacity for multiple applications.

An exemplary system for electro-stimulation, impedance monitoring and extracellular recording of excitable cells requires electrical connection of a device to a suitable a suitable electro-stimulation power source, impedance analyzer, and/or extracellular recording amplifier. With the above in mind, the invention provides a system for monitoring excitable cells, which includes a device having at least one well, each well having a bottom having a nonconductive substrate, wherein the substrate has a surface suitable for attachment of excitable cells; a power source configured to deliver an electrical signal capable of electro-stimulating excitable cells; and at least one analyzing module for measuring an electrical property from electro-stimulated excitable cells, characterized in that each well includes a pair of electro-stimulation electrodes configured to receive the electrical signal from the power source thereby delivering an electro-stimulating signal to the well for electro-stimulation of excitable cells attached to the substrate; and at least a second pair of electrodes communicatively coupled to the at least one analyzing module, which is selected from the group consisting of a pair of impedance monitoring electrodes communicatively coupled to the at least one analyzing

module in the form of an impedance analyzer thereby permitting impedance monitoring of excitable cells attached to the substrate, and an extracellular recording electrode pair communicatively coupled to the at least one analyzing module in the form of an extracellular recording amplifier thereby permitting extracellular recording of excitable
5 cells attached to the substrate.

Referring to **FIG. 3**, in some embodiments a the power source **220**, impedance analyzer **230** and extracellular recording amplifier **240** are combined into a combined analyzing module **200**, which is operably connected to a multiwall device **100** for electro-stimulation and monitoring of excitable cells. An electronic switch **210** can selectively
10 switch electrical connection with at least one electrode of the device **100** between the power source **220** and impedance analyzer **230**, between the power source **220** and extracellular recording amplifier **240**, and/or the impedance analyzer **230** and extracellular recording amplifier **240**, thereby providing numerous combinations. The combined analyzing module **200** can then be coupled to a computer **300** loaded with
15 software to execute programming or analyze data.

Accordingly, in some embodiments of the invention, a system for monitoring excitable cells is provided, which includes a device for electro-stimulation and monitoring of excitable cells, a power source capable of delivering an electrical signal to a pair of electro-stimulation electrodes that stimulates excitable cells; at least one
20 analyzing module in the form of an impedance analyzer capable of monitoring cell substrate impedance of cells through the pair of impedance monitoring electrodes or an extracellular recording amplifier capable of recording extracellular potentials of cells through the extracellular recording electrode pair. The system is configured to electro-stimulate the excitable cells at a different time compared to measurement from the at least
25 second pair of electrodes.

Electro-stimulation is accomplished by an electrical signal delivered from the power source to the electro-stimulation electrodes and to attached cells, which can be in the form of a series of electrical pulses. That is, while impedance monitoring and extracellular recording may incorporate different spectra, waveforms or the like that can
30 continue over an extended time, electro-stimulation is typically performed more quickly in a fast on-off approach followed by a delay in the off state to permit measurement of

the excitable cells without interference of the electro-stimulation signal. The skilled artisan will appreciate that in some instances the electro-stimulation signal may be at least partially filtered to prevent interference from a lower intensity state. Preferably, electro-stimulation of the excitable cells is performed at a plurality of time intervals. Most preferably, the time intervals are at regular time intervals. As a nonlimiting example, electro-stimulation can be performed by delivering a biphasic pulse of about 0.8-1.5 ms, more preferably about 1 ms and at an output voltage of 1-2 V or more preferably about 1.2 V.

An extracellular recording amplifier is communicatively coupled to the extracellular recording electrode pair for electrical communication to permit amplifying and recording electrical voltage signals between the recording electrodes and reference electrodes. That is, extracellular recorded voltage signals are recorded as the difference in the electrical potentials between the recording electrode and reference electrode. 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. 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 voltage signals and to ensure that the majority, if not all, of the recording voltage signals are derived from that on the recording electrode.

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. 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 extracellular 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, it may be preferable to have at least two recording electrodes positioned at about the middle of the array and in some instances more. Extra-cellular potentials from each such recording electrode can be amplified and recorded separately. The user can then pick and choose appropriate signal waveforms recorded from individual recording electrodes for data analysis. Alternatively or in combination, a preferred approach is to culture a layer of excitable cells such that the layer covers all recording electrodes.

In the system for monitoring impedance of excitable cells the impedance analyzer is communicatively coupled to impedance monitoring electrodes to monitor impedance. In some embodiments, the impedance analyzer is capable of measuring impedance between 0.1 ohm and 10^5 ohm in frequency range of 1Hz 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 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 having shorter excitation cycles or pacing electro-stimulation more quickly, 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 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 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 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.

Achieving millisecond time resolution can be achieved by using fast processing electronic chips for analogue-to-digital conversion, for parallel digital signal processing and data calculation with field-programmable gate array (FPGA) and for fast communication between the impedance measurement circuitry and software. Another example of 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 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 millisecond resolution.

In addition, when using a device having multiple wells, the software can issue a command for measuring multiple wells' impedances. The measurement 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 the same time or simultaneously with more than one well's data being sent at a time. In this "measurement of multiple-wells' impedances 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.

With millisecond time resolution for impedance measurement, it becomes possible to resolve individual beating cycles of cardiomyocytes cultured on electrodes and study the excitation, contraction and release of beating cells. 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.

Cell-substrate impedance monitoring at 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 switching between occur 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.

As discussed above, one or more electrodes in a pair of electrodes can be shared with another pair of electrodes. Sharing of electrodes can be accomplished at least in part by switching the communicative coupling for electrical connection between the power source for electro-stimulation and either the impedance analyzer or extracellular recording amplifier. Alternatively, sharing of electrodes can be accomplished at least in part by switching the communicative coupling for electronic connection between the impedance analyzer and the extracellular recording amplifier. In each instance, switching is preferably performed through an electronic programmable switch and is provided together with the power source, impedance analyzer and extracellular recording amplifier in a combined analyzer, which itself can be coupled to a computer for performing measurement instructions or data analysis.

In some embodiments a combined analyzer 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, such as in the form of a device station with carriage. The one or more platforms or one or more slots can
5 comprise sockets, pins or other devices for electrically connecting the device to the device station such as interaction through connection pads. The system can be configured such that multiwell devices can be positioned in a tissue culture incubator during electro-stimulation, extracellular recording or impedance monitoring while the computer and optionally combined analyzer are located outside the tissue culture incubator. This can
10 be accomplished through the electrical connection of the device through a device station that itself is inside the incubator and electrically coupled to a combined analyzer that is outside of the incubator. Alternatively, the combined analyzer can be within the incubator and communicate with the computer that is outside of the incubator.

The device station or electromechanical apparatus or assembly capable of
15 interfacing multiwell devices includes electronic circuitry that connect to the power source, impedance analyzer, and/or extracellular recording amplifier and can incorporate electronic switches that can switch on and off connections to each of the two or more pairs of electrodes within a well of the multiwell devices used in the system. The switches of the device station or electromechanical apparatus or assembly capable of
20 interfacing multiwell devices are controlled by a software program, each of which preferably provides millisecond time resolution.

The systems of the invention are typically controlled through commands sent from a computer operably connected to the power source, impedance analyzer and extracellular recording amplifier. The computer is typically loaded with software that
25 provides two functions. A first is data acquisition and a second is data analysis. The software uses graphical interfaces for ease of use.

The data acquisition software is used to define the experiment, setup recording steps, monitor the raw data, adjust settings and to collect raw data files. An example of graphical interface for data acquisition is shown in **FIGS. 4A-C**, where **FIG. 4A** shows
30 an interface for entering experimental notes such as experiment name, purpose or the like; **FIG. 4B** shows a well layout where HL-1 atria cells are seeded at different density

from 10,000 in column 1 to 40,000 in column 4, and **FIG. 4C** shows wells specific settings for, electro-stimulation, impedance monitoring and extracellular recording.

Preferably, the software can also analyze impedance data and extracellular recording data. An exemplary graphical interface for data analysis is provided in **FIG. 4D**, which shows a file structure “tree” at the left side for file management, single or multiple channels of raw data file displayed in the middle section of the window and the analysis result displayed at the right side of the window. In addition, the data analysis software also has the ability to do single field potential waveform analysis.

An overview of an exemplary experiment and how it interacts with the software can be seen in **FIG. 5**, where a first step involves cell preparation **400**, such as culturing cells, proceeds through raw data collection **500** using electro-stimulation **510** if extracellular recording or impedance monitoring does not result in desired activity, such as poor beating of cardiomyocytes. After raw data collection **500** data analysis **600** is conducted such as correlation of Impedance and ECR data **620**. Finally reports **700** are generated providing the results from the impedance and extracellular recording results in desired formats.

In preferred embodiments, the software can calculate an impedance-based parameter, such as cell index (CI) for one or more time points for one or more wells of the multiwell device through suitable programming. In some preferred embodiments, the software can also calculate a cell change index (CCI) from impedance measurements of one or more wells of the multiwell device. The software can preferably generate plots of impedance-based parameters over time, such as but not limited to impedance-based curves selected from impedance measurements, CI, or CCI. The software may perform other analysis as well, such as calculate cell number from CI, generate dose-response curves based on impedance data, calculate IC values based on impedance values, and calculate kinetic parameters of the excitation cycle cell based on impedance-based parameters and impedance-based curves. In some embodiments the beating cycle of a cardiomyocyte population is determined, which may include initiation and decay of individual beats. Peaks may be derived from the detection of vectors associated with initiation of beating or beating decay. Peaks may be derived with other methods. In further embodiments the change in beating cycle of a cardiomyocyte population is

determined in response to a stimulus such as administration of a compound to the cells. The software of the impedance monitoring system can also store and display analyses of the data, such as calculated impedance values impedance-based parameters and kinetic parameters derived therefrom, Data can be displayed on a screen, as printed data, or both.

5 Data may be stored on a hard drive for exportation into compatible programs for further analysis or data storage

10

Methods of Monitoring Cardiomyocyte Beating, Viability, Morphology and Electrophysiological Properties

The invention provides methods of monitoring excitable cells, methods for

15 monitoring cardiomyocyte beating, viability, morphology, assessing cardiotoxic affects associated with administering of one or more compounds to a population of cardiomyocyte or cardiomyocyte precursor cells. The methods include obtaining a sample of excitable cells, electro-stimulating the excitable cells; and impedance monitoring and/or extracellular recording of the excitable cells. Preferably, impedance

20 monitoring and extracellular recording is performed simultaneously to achieve simultaneous real time measurements.

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. The methods and systems herein have developed a system

25 for monitoring cell substrate impedance to precisely measure and quantify these changes in cell morphology and shape.

In addition, the present invention also discloses an approach for monitoring extracellular field potential, which is an electrical potential produce by cells that is outside of the cell. However, as shown in the examples, extracellular recording also

30 provides insight as the activity of ion channels, which are of central interest when assessing cardiotoxicity of potential therapeutics.

In view of the above, a method of monitoring excitable cells is provide, which includes providing a system for electro-stimulation, impedance monitoring and extracellular recording of excitable cells; adding a sample of excitable cells to the device; electro-stimulating the excitable cells; and impedance monitoring and extracellular
5 recording of the excitable cells.

In a related embodiment, a method of monitoring excitable cells is provided, which includes providing a system for electro-stimulation and impedance monitoring of excitable cells; adding a sample of excitable cells to the device; electro-stimulating the excitable cells; and performing impedance measurements of the excitable cells.

10 In another related embodiment, a method of monitoring excitable cells is provided, which includes providing a system for electro-stimulation and extracellular recording of excitable cells; adding a sample of excitable cells to the device; electro-stimulating the excitable cells; and performing extracellular recording of the excitable cells.

15 Among the cells that may be monitored include any excitable cells, such as primary cardiomyocytes harvested from animal tissue, cultured cardiomyocytes, cardiomyocyte precursor cells, embryonic stem cells, embryonic stem cell derived cardiomyocytes, cardiomyocyte cell lines, enriched cardiomyocyte cells and the like. Alternatively the invention has also shown that neuronal cells may also be used.

20 Obtaining or harvesting suitable excitable cells may be performed using a variety of harvesting, collecting, culturing, purifying, enriching or differentiating techniques known in the art to which the invention belongs. While the device can detect changes, such as impedance change and extracellular potential in a single cell, preferably samples contain a plurality of cells so that the cells will join together for communication with one
25 another, such as through gap junctions. Clear changes in electrocellular recording and impedance measurement have been identified using population of about 10,000-14,000 cells; however, this is not intended to be limiting.

Cells may be placed into culture then immediately stimulated or measured; however, it has generally been preferable to culture cells such that they attach to the
30 substrate prior to stimulation. More preferably, cells are grown or differentiated on the substrate for a sufficient time in that they join to form a cell layer over the recording

electrodes of the extracellular recording electrode pair and over the pair of impedance monitoring electrodes. In some embodiments, electro-stimulation is performed within 1 hour of placing cells in the device; however, the substrate forming the bottom of the well has been shown suitable for long term culturing and thus cells may be cultured as desired, such as for more than 5 days, more than 10 days, more than 15 days, more than 20 days or even more than one month depending on the interests of the experiment and regular cell culture maintenance.

Further, impedance monitoring may be used to assess how well cells are attached to the substrate, the viability of the cells or to assess the quantity of cells in the sample prior to electro-stimulation, to assess growth curves or the like. Further, impedance or extracellular recording may be used to determine whether electro-stimulation is necessary or desired. In some instances cardiomyocytes derived from embryonic stem cells will begin to beat spontaneously, which can be detected or measured through impedance and extracellular recording. However, even cells that being spontaneous beating can be electro-stimulated to establish a more regular beating or a beating at an increased frequency.

Excitable cells are electro-stimulated through applying a signal via the pair of electro-stimulation electrodes. As indicated above, the pair of electro-stimulation electrodes may be a dedicated pair of electrodes, preferably one or more electrodes within the pair of electro-stimulation electrodes may be shared between the pair of impedance measurement electrodes or the extracellular recording electrode pair. Sharing of the electro-stimulation function is permitted in part because of the fast switching between connectivity to the power source for electro-stimulation and the impedance analyzer or between the power source for electro-stimulation and the extracellular recording amplifier. While the particular specifications of a device can vary, stimulation is generally accomplished by applying voltage in a range of about 1 – 2 V for about 1ms, at an interval of every 1-1.5 seconds or so. Particularly encouraging results were obtained when applying 1.1 V for 1 ms every 1.2-1.3s.

When providing two or more wells, each well may be pulsed together such that electro-stimulation occurs simultaneously in more than one well or pulses may be

staggered such that two or more wells are pulsed with different at different interval. Electro-stimulation intervals can be selected through a software and computer interface.

When performing impedance monitoring, preferably, impedance is measured at millisecond time resolution. For example, resolution on the order of 1-10 ms provides
5 high resolution of beating of cardiomyocytes. Impedance measurements may themselves be compared; however, in preferred embodiments a cell index is calculated from impedance measurements as an impedance parameter and plotted over time as an impedance-based curve or cell index curve. Accordingly, a cell index curve may be used as an impedance-based curve for comparison, such as by comparing cell index curves
10 over time. In other embodiments, cell change index is calculated from cell index and plotted as an impedance based curve. Accordingly, cell change index may be used as impedance-based parameter for comparison, such as by comparing cell change index curves over time. The skilled artisan will appreciate that when comparing impedance-based curves, a same type of curve will be compared. For example, comparison would
15 involve comparing cell index curves to one another, cell change index curves to one another and the like.

While impedance measurements themselves can be compared, their comparison is complicated by culture conditions, such as differences between cell populations. Accordingly a number of calculations have been established previously that result in
20 values that permit improved comparison. Among these are cell index (CI) and cell change index (CCI), each of which has been discussed in detail in the following US Patents: US 7,470,533, US 7,459,303, US 7,192,752, US 8,026,080, US 7,560,269, US 8,263,375, US 7,468,255,, US 8,206,903 and US 8,420,363. Accordingly, the use of cell index and cell change index in the formation of an impedance-based curve is well
25 established in the art. Further, these documents can be consulted for method of calculating cell index (CI) or cell change index (CCI). However, a brief overview introduction is provided below.

The cell index (CI) obtained for a given well reflects how many cells are attached to the electrode surfaces in this well and how well cells are attached to the electrode
30 surfaces in the well. In this case, a zero or near-zero "cell 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 cell index =0. A higher value of “cell index” indicates that, for 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 same type of the cells and same number of the cells, cells are attached better (for example, cells spread out more, or cell adhesion to the electrode surface is stronger) on the electrode surfaces.

In other embodiments, a normalized cell index is calculated from the cell index and plotted as an impedance based curve. 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.

In other embodiments, a cell change index (CCI) is calculated from the cell index. A “cell change 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 cell change index is the absolute change in the cell index from an initial time (the standard time point) to the measurement time. CCI is the normalized rate of change in cell index. CCI values can be used to quantify the cell status change. For cells in an exponential growth under regular cell culture condition, the cell index determined by a cell-substrate impedance monitoring system described herein is expected to be a proportionate measure of the cell number in the well since the cell morphology and average extent of cell adhesion to the electrode surfaces among the whole cell population do not exhibit significant changes over time.

Turning back to comparing impedance based parameters or impedance based curves, in preferred embodiments, cell index is preferably calculated and plotted over time to form an impedance base curve. Impedance based curves over time may be aligned or overlayed with one another according to electro-stimulation time points. For example, two or more impedance-based curves may be aligned or overlayed using a point or time of electro-stimulation as a starting basis.

The skilled artisan will appreciate that when comparing impedance-based parameters each member for comparison is a member of a same parameter. That is, impedance measurements can be compared to one another; cell index, which can be calculated from the impedance measurement, can be compared to one another; or cell change index, which can be calculated from cell index, can be compared to one another. Although individual members of a parameter can be compared, preferably the impedance based parameters are plotted over time to provide an impedance-based curve then compared, whether an impedance measurement curve, a cell index curve, or cell change index curve to identify differences changes in impedance, which may be associated with administration of a compound, expression of an inserted nucleic acid or the like. In preferred embodiments, cell index is calculate from impedance measurements and plotted over time to provide an impedance-based curve in the form of a cell index curve.

Extracellular recording measures the extracellular potential (also referred to as field potential (FP)) and is a close simulation to its counterpart, intracellular action potential (AP), therefore identifying peaks and wave form changes in field potential can be used to predict ion channel activity. Accordingly, the extracellular recording measurements are plotted over time, typically in microvolts, to identify variations in the field potential (FP). Sampling rate for extracellular recording can be about 1KHz, 2 KHz, 5KHz and 10 KHz. Extracellular potential is preferably plotted over time to form a field potential curve.

Periods or electro-stimulation intervals from two or more impedance curves and/or two or more field potential curves may be overlayed to identify trends or differences in impedance and/or field potential. For clarity, the two or more impedance curves can be from different wells, such as from wells having serial dilutions of a compound or may be from a same well at a later time point. Accordingly, the addition of

compounds suspected of affecting either the field potential (likely due to affecting ion channels) or impedance of the excitable cells may be added to a culture of cells and changes in impedance or field potential can be identified or measured through the comparison of the an impedance-based parameter and field potential before and after compound addition, such as by comparing cell index curves or field potential curves over time. Accordingly, in preferred embodiments comparisons are performed by comparing cell index curves over time to one another and comparing field potential curves over time to one another. In instances where it is not readily apparent by viewing each curve over time, curves between different electro-stimulation periods can be overlaid and compared using curve comparison algorithms. Accordingly, overlaying impedance curves may identify changes in impedance and overlaying field potential curves may identify changes in field potential.

The methods are useful in assessing the cardiotoxicity of compounds through monitoring impedance of a beating cell population and identifying any changes after compound administration. For instance, a compound suspected of affecting excitation contraction coupling of the excitable cells can be provided to the well. Impedance monitoring can be performed before and after adding the compound and impedance-based parameters (such as cell index) prior to and after adding the compound can be calculated and compared to identify changes in the impedance parameter in response to the compound and thus predict whether the compound is likely to be cardiotoxic. Preferably, the impedance based parameter is compared between at least two different electro-stimulation intervals; however, three or more, four or more, five or more, six or more and the like intervals can be compared.

In some embodiments, impedance and/or extracellular recording is conducted to assess an effect of a compound, such as its potential cardiotoxicity, on cell. An exemplary method would be to provide a culture of cells, electro-stimulate the cells, perform impedance monitoring and/or extracellular recording before and after adding the compound and analyzing the results. As such, the analysis could include calculating an impedance-based parameter (such as cell index or cell change index) from impedance prior to and after adding the compound and their comparison to identify changes in the impedance parameter. Further, the impedance-based parameter may be plotted over time

to form an impedance-based curve before and after administration for comparison. Alternatively or in addition, extracellular potential before and after administration can be compared such as by comparing field potential curves over time to identify differences. Such analysis in response to the compound can be used to predict whether the compound is likely to be cardiotoxic, affects beating, affects cardiomyocyte morphology or affects ion channels. Preferably, the impedance based parameter is compared between at least two different electro-stimulation intervals; however, three or more, four or more, five or more, six or more and the like intervals can be compared. Compounds for testing are not intended to be limiting and may include organic or inorganic molecules, drugs, peptides, proteins, antibodies, siRNA, shRNA, miRNA, cDNA, lipids or any combination thereof.

While the above has been primarily discussed with respect to monitoring the excitable cells before and after adding a compound suspected of having a cardiotoxic affect to a well and comparison of impedance or field potential before and after administration, in related approaches the compound can be added to two or more wells in different concentrations to evaluate cardiotoxicity or activity compared to dose or can be added to two or more wells and administered in same well with an antagonist, such as to further verify suspected findings or mechanism of action. For instance, impedance-based curves can be used to calculate the compound dose-dependent changes in cardiomyocyte morphology, ion channel modulation, impedance, field potential or the like 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.

Still further, analysis of impedance curves or extracellular recording curves after compound administration can be clustered and grouped according to similarities thereby being suggestive of a common mechanism of action. Further, once profiles of known compounds are grouped, unknown compounds can then be tested in such a system, and the impedance-based curves and extracellular recording curves compared to the group, then the compound can be assigned to one or more group based on similarities or differences between curves.

In view of the above description and the examples that follow, it should become evident that while systems of the invention can be applied for cardiac electrophysiology

recording for drug/compound profiling and cardiac safety screening, it can also be used to record from any excitable preparations. With incorporation of cell electro-stimulation, field potential recording and cell-impedance monitoring technologies, this system is the first single instrument which can simultaneously monitor the cardiac excitation by field potential and beating functioning by impedance *in vitro*, yet the device still adds electro-stimulation of cells to induce or regulate excitation contraction coupling or beating of a cell population and thus permits use of cells that require stimulation to begin or continue beating.

10 Assessing Genetically Manipulated Embryonic Stem Cells and Effects on Cardiomyocyte Function

In another aspect, the present invention is directed to method to assess the effect of gene knockout in an embryonic stem (ES) cell upon differentiating into to a cardiomyocyte, the method including providing a device for monitoring impedance operably connected to an impedance analyzer, wherein the device includes at least two wells; adding wildtype ES cells as control to at least a first well and ES cells with a gene knockout in at least a second well; optionally electro-stimulating cells in each well; monitoring impedance of the at least two wells and optionally determining cell indices from impedance values to generate an impedance-based curve for each well; comparing the impedance-based curves between the first and second wells, and if significantly different, concluding that the gene knockout may affect at least one selected from the group consisting of cardiomyocyte viability, cardiomyocyte morphology, cardiomyocyte beating.

25 In a related approach, the invention also provides a method to assess the effect of gene knockout in an embryonic stem (ES) cell upon differentiating into to a cardiomyocyte, the method including: providing a device for monitoring impedance and extracellular recording, that are operably connected to an impedance analyzer and extracellular recording amplifier, wherein the device includes at least two wells; adding wildtype ES cells as control to at least a first well and ES cells with a gene knockout in at least a second well; monitoring impedance and extracellular recording of the at least two

wells and optionally determining cell indices from impedance values to generate an impedance-based curve for each well; comparing the impedance-based curves and field potential curves between the first and second wells, and if significantly different, concluding that the gene knockout may affect at least one selected from the group consisting of cardiomyocyte viability, cardiomyocyte morphology, cardiomyocyte beating or affecting an electrophysiological property of the cardiomyocyte.

In another aspect, the present invention is directed to method to assess the effect of a transgene in an embryonic stem (ES) cell upon differentiating into to a cardiomyocyte, the method including providing a device for monitoring impedance operably connected to an impedance analyzer, wherein the device includes at least two wells; adding wildtype ES cells as control to at least a first well and ES cells harboring a transgene knockout in at least a second well; optionally electro-stimulating cells in each well; monitoring impedance of the at least two wells and optionally determining cell indices from impedance values to generate an impedance-based curve for each well; comparing the impedance-based curves between the first and second wells, and if significantly different, concluding that the transgene may affect at least one selected from the group consisting of cardiomyocyte viability, cardiomyocyte morphology, cardiomyocyte beating.

In a related approach, the invention also provides a method to assess the effect of a transgene in an embryonic stem (ES) cell upon differentiating into to a cardiomyocyte, the method including: providing a device for monitoring impedance and extracellular recording, that are operably connected to an impedance analyzer and extracellular recording amplifier, wherein the device includes at least two wells; adding wildtype ES cells as control to at least a first well and ES cells harboring a transgene in at least a second well; monitoring impedance and extracellular recording of the at least two wells and optionally determining cell indices from impedance values to generate an impedance-based curve for each well; comparing the impedance-based curves and field potential curves between the first and second wells, and if significantly different, concluding that the transgene may affect at least one selected from the group consisting of cardiomyocyte viability, cardiomyocyte morphology, cardiomyocyte beating or affecting an electrophysiological property of the cardiomyocyte.

EXAMPLES

Example 1

Specifications of an Exemplary System for Electro-Stimulation, Impedance Measurement and Extracellular recording

A system was constructed for electro-stimulation of excitable cells, as well as for impedance measurement and extracellular recording of excitable cells having two electrodes for extracellular recording, and shared electrodes in the form of a pair of interdigitated electrode structures having a circle on line configuration that function both as electro-stimulation electrodes and impedance monitoring electrodes. The system was designed with the following specifications. The hardware system bandwidth was 1Hz~2KHz (-3dB). The suppression ratio of impedance stimulation signal was > 60 dB. The noise level was 10 μ V (Vp-p). The system had excellent shielding and electromagnetic compatibility (EMC).

In regards to the electro-stimulation signal available waveforms are rectangular form, a ramp form, and sinusoidal. The polarity can be set for uni-polarity or bi-polarity. An exemplary amplitude is -2.5V~+2.5V@ $\pm$ 100mA per channel. An exemplary output voltage resolution is 2mV. An exemplary rise time (10 to 90 %) of voltage: 0.2 μ s @ $\Delta U=2$ V. An exemplary time lag between stimulation and voltage output is $5 \pm 1\mu$ s @ amplitude > 200mV. An exemplary current output is -250 mA ~ +250mA@per channel. An exemplary resolution is 12 bit. An exemplary time resolution is 10 μ S. A maximum frequency (rectangular waveform) is 50 KHz. A stimulation format can stimulate all 96 wells in 96 well plate simultaneously or by column (8 wells). Further, in some instances impedance and extracellular recording measurement could be conducted simultaneously with stimulation.

The sampling rate for impedance measurement over 96 wells was < 10 ms. The sampling rate for extracellular recording signal was 1 KHz, 2 KHz, 5 KHz, and 10 KHz. Maximal sampling channels for ECR signal was maximally 8 channels (wells) in parallel, recorded from either one of two electrocellular recording electrodes in a single well.

Data acquisition mode permits extracellular recording only, impedance measurement only, and extracellular recording and impedance measurement simultaneously.

A gain setting offers 1 K, 2 K, 4 K, and 8 K. ADC resolution is 14 bit/ $\pm 5V$, total AD conversion rate of 800K SPS. Temperature rise in a well was lower than 0.4C temperature rise compared to incubator temperature. DC offset is fixed at 0.05Hz, $\pm 1mV$. Input voltage range is $\pm 2.5 mV$. Electrode input impedance of gold having a diameter 60 μm /100 μm is $< 300K\Omega / 1 KHz$

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Example 2

Monitoring the Spontaneous Field Potential Firing and Changes in Impedance of Cultured Cells

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HL-1 atria cells were harvested and cultured in wells having a pair of extracellular recording electrodes. **FIG. 6A** shows results from eight paralleled ECR signals in the HL-1 atria cells at day 7 with 40,000 cells per well, some channels showed spontaneous field potential (FP) firing but no impedance (IMP) signals. As can be seen, the firing is not paced at a regular rate.

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CorAT cells (mouse embryonic stem cell derived cardiomyocytes) were cultured over time. At day 6, with a cell density at 45,000 per well, spontaneous field potential (FP) firing was detected as well as changes in impedance (IMP) as shown in **FIG. 6B**.

While the above experiments demonstrate the usefulness of ECR and impedance monitoring of cells, spontaneous firing and impedance changes were detected at different time points across different samples

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Example 3

Modulation of Ion Channels while Performing Extracellular Recording and Impedance Monitoring

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CorAt cells were cultured over time and ECR and IMP signals were recorded simultaneously. Different doses of Sotalol were added at day 9 with CorAt cells at a density of 40,000 cells per well. **FIGS. 7B-C** show results from 200 μ M and 400 μ M. **FIG. 5A** is control without Sotalol, **FIG. 5B** shows treated wells or test wells with 200 μ M, and **FIG. 5C** shows treated wells or test wells with 400 μ M.

Administration of 400 μ M sotalol induced a typical EAD and arrhythmias which indicating the primarily target of sotalol is the IKr (hERG) as it was well characterized and published in literature. The other ion channels (INa and ICa) were secondarily inhibited; this may due the delay of recovery of membrane potential repolarization, so the available portion of excitable INa and ICa channels were reduced.

Primary rat cardiomyocytes were cultured over time and ECR and IMP signals were recorded simultaneously. Different doses of Quinidine were added at day 2 with the cardiomyocytes at 20, 000 cells per well. **FIG. 8A** shows an ECR and IMP overlay with Quinidine added at 0 μ M. **FIG. 8B** shows an ECR and IMP overlay with Quinidine added at 2 μ M. **FIG 8C** shows an ECR and IMP overlay with Quinidine added at 4 μ M. **FIG. 8D** shows an ECR and IMP overlay with Quinidine added at 8 μ M. **FIG. 8E** shows an ECR and IMP overlay with Quinidine added at 16 μ M. **FIG. 8F** shows an ECR overlay of from **FIGS. 8A-E**. **FIG. 8G** shows an overlay of impedance-based curves of **FIGS. 8A-E**.

Example 4

Field Stimulation and pacing of mouse embryonic stem cell derived cardiomyocytes (Cor.AT) using the RTCA Cardio-ECR System

Cor.AT cells are mouse embryonic stem cell derived cardiomyocytes that are engineered to express the GFP protein and puromycin protein for selection. These cells are a 100% pure population of cardiomyocytes that beat spontaneously in culture and express all the repertoire of ionic currents typical of a normal cardiomyocytes such as calcium, potassium and sodium.

To assess the field stimulation and pacing of Cor.AT cells, the cells were seeded in the wells of Cardio-ECR plates coated with fibronectin at 10 ug/mL and at a density of

35,000 cells/ well. This density has been empirically optimized to sustain a cell monolayer that can beat in a synchronous manner. The cells were allowed to form a monolayer and the media was changed to fresh media on daily basis. On the 9th day after seeding the cells were subjected to field stimulation. The cardiomyocytes were field
5 stimulated at 1.2 second duration with a biphasic pulse of 1 ms total length and an output voltage of 1.05 V. The total duration of stimulation was for 40 seconds.

Both the field potential and impedance signals were dynamically monitored by the Cardio-ECR System. As shown in **FIG. 9**, application of the stimulation pulse caused regular pacing of the Cor.AT cells as measured primarily by the impedance signal (Red
10 Trace) which signifies contraction of the cardiomyocytes. The actual ECR signal during the stimulation phase (Blue Trace) is overwhelmed by the field stimulation signal but can be observed when there is no stimulation.

15 Example 5

Field Stimulation and Pacing of Rat Neonatal Cardiomyocytes

Using the RTCA Cardio-ECR System

Rat neonatal primary cardiomyocytes are spontaneously beating cardiomyocytes
20 that are used as a model system for studying heart function. This model is well-established for the study of the transport and toxicity of drugs and electrophysiological characterization. Neonatal rat cardiomyocytes are prepared and harvested as follows:

Briefly, hearts from neonatal rats are rapidly excised and washed to remove blood and debris. The ventricles are carefully minced and dissociated into single cells by
25 proteolytic enzymes during repetitive digestions with gentle stirring. Suspending cells in serum containing growth medium terminate the proteolytic digestion. Primary cardiomyocytes are purified and enriched by pre-plating twice for 45 minutes each time. The cells are counted and adjusted to a desirable concentration and then directly seeded
30 in the wells of the Cardio-ECR Plates coated with 0.1% gelatin at a density of 14,000 cells/ well. This density has been empirically optimized to sustain a cell monolayer that can beat in a synchronous manner. The cells were allowed to form a monolayer and the media was changed to fresh media on daily basis. On the 5th day after seeding the cells

were subjected to field stimulation. The cardiomyocytes were field stimulated at 0.8 second duration with a biphasic pulse of 1.0 ms total length and an output voltage of 1.2V.

Both the field potential and impedance signals were dynamically monitored by the Cardio-ECR System. As shown in **FIG. 10**, application of the stimulation pulse caused regular pacing of the rat neonatal primary cells as measured primarily by the impedance signal (Red Trace) which signifies contraction of the cardiomyocytes. The actual ECR signal during the stimulation phase (Blue Trace) is overwhelmed by the field stimulation signal but can be observed when there is no stimulation.

Example 6

Electro-stimulation Coupled with Extracellular Recording Shows Change in Field Potentials when adding Different Concentrations of the Calcium Channel Blocker Isradipine

To show the benefit of coupling electro-stimulation with extracellular recording, impedance and extracellular recording measurements were performed on HL-1 cells. Electro-stimulation was then applied at electro-stimulation parameters of 1.1 V intensity, 1ms pulse width, 1.3 s stimulation interval. As shown in **FIG. 11A**, prior to electro-stimulation, the HL-1 cells showed no measureable changes in impedance (IMP) or extracellular recording field potential (FP); however, **FIG. 11B** shows electro-stimulation induced FP in the extracellular recording measurement and thus a “waking up” of the cells.

Cells were then treated with Isradipine, a L type Ca^{2+} channel blocker at either 0.25 μM (**FIG. 11C**), 0.5 μM (**FIG. 11D**), or 1 μM (**FIG. 11E**). **FIG. 11F** shows an overlay of the extracellular recording field potentials. By application of the different doses of isradipine, the slow component of the induced FP (Ca channel) was gradually inhibited.

Example 7

Electro-stimulation Coupled with Extracellular Recording Shows Change in Field Potentials when adding Different Concentrations of the Calcium Channel Activator Bay K8644 and Addition of Antagonist

As another example demonstrating the benefit of coupling electro-stimulation with extracellular recording, impedance and extracellular recording measurements were performed on HL-1 cells. HL-1 cells were cultured on a device having a pair of electro-stimulation electrodes, a pair of impedance measurement electrodes and a recording and reference electrode pair. Electro-stimulation was then applied at electro-stimulation parameters of 1.1 V intensity, 1ms pulse width, 1.3 s stimulation interval. As shown in **FIG. 12A**, prior to electro-stimulation, the HL-1 cells showed no measureable changes in impedance (IMP) or extracellular recording field potential (FP); however, **FIG. 12B** shows electro-stimulation induced FP in the extracellular recording measurement and thus a “waking up” of the cells followed by lack of FP after stopping electro-stimulation.

Cells were then treated with Bay K8644, a L type calcium channel activator at 1 μM (**FIG. 12C**). The spontaneous firing rate was increased due to K8644 application, but its firing is irregular. The cells were then electro-stimulation to pace the firing with the K8644 still in the medium using the electro-stimulation parameters of 1.1 V, 1ms, 1.3s as shown in **FIG. 12D**. The spontaneous firing rhythm was gradually adapted to the stimulation frequency.

Cells were then treated with Nifedipine to antagonize the effect of Bay K8644 at either 0.25 μM (**FIG. 12E**) or 0.5 μM (**FIG. 12F**). The stimulation induced firing rate was remarkably inhibited due to nifedipine application. **FIG. 12G** shows the overlap of the field potential traces from **FIGS. 12A-F**. Based on the overlapped trace analysis, we can see that the FP was dose dependently inhibited by L type Ca^{2+} channel blocker, Nifedipine, the inhibition effect was mainly attributed to the blocking the Ca^{2+} peak (slow component followed by the initial fast Na^{+} peak).

Example 8

Electro-Stimulation of CorAT cells and the Pacing of Cardiomyocytes

CorAT cells were cultured on a device having a pair of electro-stimulation electrodes, a pair of impedance measurement electrodes and a recording and reference electrode pair. At day 9 CorAT cells with a density of 35,000 per well were paced by applying 1.05 V at every 1.2 seconds for a duration of about 40 seconds. As shown in **FIGS. 13A-B**, pacing was induced to produce reproducible plots of both impedance (shown in cell index) and extracellular field potential; however, when pacing by electro-stimulation stopped, both impedance and field potential were irregular. In **FIG. 13A**, impedance and field potential are overlaid whereas in **FIG. 13B** impedance is the upper display and extracellular recording of field potential the lower.

FIG. 13C shows a single interval of electro-stimulation showing a relationship between change in impedance (top) and extracellular recording field potential (lower)

Example 9

Electro-Stimulation of Rat Primary Cardiomyocytes and the Pacing of Cardiomyocytes

Rat primary cardiomyocytes were harvested and cultured on a device having a pair of electro-stimulation electrodes, a pair of impedance measurement electrodes and a recording and reference electrode pair. At day 5, the cells had a cell density of 14K per well.

Cells were paced by applying electro-stimulation (1.2V, 1ms, 0.8 s, stimulation duration: ~40 s). **FIGS. 14A-B** showed the regular pacing of the cardiomyocytes and the reproducible impedance and field potential responses; however, when pacing stopped the regular impedance and field potential profiles also stopped. **FIG. 14A** shows impedance as an overlay with field potential while **FIG. 14B** shows impedance on the top panel and field potential on the lower panel.

Example 10

Recording Neuronal Activity Through the Recording of Field Potential

Our cardio ECR device includes a pair of extracellular recording electrodes, the size of which resemble that of popular multichannel electrode recording. We found that our electrode configurations could be used for recording of changes in field potential (FP) from cultured neuronal network (see example data below).

Dissociated neurons from brain or spinal tissue are seeded in the well of our cardio ECR device, and allowed to mature over the course of three weeks into self-organized networks complete with axons, dendrites and hundreds to thousands of synaptic connections. The neuronal networks exhibit spontaneous bursting activity after two weeks of incubation, and the spontaneous activity could be monitored by the either one of the two ECR electrodes.

The neuronal firing data was recorded by either one of the two ECR electrodes in single well. After performing measurements from the ECR electrodes, Bicuculline, a GABAAR inhibitor, was added to the culture. As shown in **FIG. 15A**, a remarkable increased the FP spontaneous firing frequency in cultured rat cortical neurons was measured on day three. This result is consistent with a previous publication that bath application of bicuculline caused large increases in spontaneous spike firing frequency (Ma YL, Weston SE, Whalley BJ, Stephens GJ. The phytocannabinoid Delta(9)-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum. Br J Pharmacol. 2008 May;154(1):204-15.

As a complementary experiment, Glutamate was added to the culture rat cortical neurons and the field potential monitored. As shown in **FIG. 15B**, Glutamate, a non-selective GluR agonist remarkably increased the FP spontaneous firing amplitude and frequency in rat cultured cortical neurons on day ten.

CLAIMS

What is claimed is:

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1. A system for monitoring excitable cells comprising a device comprising at least one well, each well having a bottom comprising a nonconductive substrate, wherein the substrate has a surface suitable for attachment of excitable cells; a power source configured to deliver an electrical signal capable of electro-stimulating excitable cells; and at least one analyzing module for measuring an electrical property from electro-stimulated excitable cells, characterized in that each well comprises:

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a pair of electro-stimulation electrodes configured to receive the electrical signal from the power source thereby delivering an electro-stimulating signal to the well for electro-stimulation of excitable cells attached to the substrate; and

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at least a second pair of electrodes communicatively coupled to the at least one analyzing module, which is selected from the group consisting of a pair of impedance monitoring electrodes communicatively coupled to the at least one analyzing module in the form of an impedance analyzer thereby permitting impedance monitoring of excitable cells attached to the substrate, and an extracellular recording electrode pair communicatively coupled to the at least one analyzing module in the form of an extracellular recording amplifier thereby permitting extracellular recording of excitable cells attached to the substrate.

30

2. The system according to claim 1, wherein the electrical signal is a series of pulses at a regular time interval.

3. The system according to claim 2, wherein the regular time interval is from 0.5 seconds to 2 seconds.

4. The system according to claim 1, wherein the electrical signal is 1 V to 2.5 V for 0.5-2 milliseconds.

5. The system according to claim 1, wherein a percentage of a surface area of the bottom of the at least one well occupied by the pair of electro-stimulation electrodes is selected from the group consisting of 5% or more, 10% or more, 20% or more, 30% or more, 50% or more, and 70% or more.

6. The system according to claim 1, wherein each of the electro-stimulation electrodes independently comprises an unbranched electrode structure or a branched electrode structure.

7. The system according to claim 1, wherein the at least second pair of electrodes is the pair of impedance monitoring electrodes and the analyzing module is in the form of the impedance analyzer.

8. The system according to claim 7, wherein the pair of impedance monitoring electrodes is a pair of interdigitated electrode structures, wherein each electrode structure comprises a plurality of electrode elements.

9. The system according to claim 7, wherein the pair of impedance monitoring electrodes is a pair of electrode structures having a same surface area.

10. The system according to claim 7, the system comprising the extracellular recording electrode pair as a third pair of electrodes.

11. The system according to claim 10, wherein at least one electrode of the pair of electro-stimulation electrodes is also at least one electrode of the extracellular recording electrode pair thereby permitting electro-stimulation of excitable cells and extracellular recording of attached cells using a same electrode at different time points.

12. The system according to claim 1, wherein the impedance analyzer monitors impedance at millisecond time resolution.

5 13. The system according to claim 1, wherein the at least second pair of electrodes is the extracellular recording electrode pair and the analyzing module is in the form of the extracellular recording amplifier.

10 14. The system according to claim 13, wherein the extracellular recording electrode pair comprises a recording electrode and a reference electrode, wherein the recording electrode has a diameter from about 10 μm to about 200 μm or from about 30 μm to about 100 μm .

15 15. The system according to claim 13, wherein the extracellular recording electrode pair comprises a recording electrode and a reference electrode, further wherein a ratio of the area of reference electrode to the area of recording electrode is selected from the group consisting of 2 or more, 10 or more, 100 or more, 1,000 or more, and 10,000 or more.

20 16. The system according to claim 13, further comprising a second recording electrode having a same diameter as a first.

25 17. The system according to claim 13, wherein at least one electrode of the pair of electro-stimulation electrodes is also at least one electrode of the pair of impedance monitoring electrodes thereby permitting electro-stimulation of excitable cells and impedance monitoring of attached cells using a same electrode at different time points.

30 18. The system according to claim 17, wherein the pair of impedance monitoring electrodes is a pair of interdigitated electrode structures, wherein each electrode structure comprises a plurality of electrode elements.

19. The system according to claim 18, wherein at least one electrode of the pair of impedance monitoring electrodes is the reference electrode.

20. A device for monitoring excitable cells, the device comprising:

5

a) at least one well, each well having a bottom comprising a nonconductive substrate, wherein the substrate has a surface suitable for attachment of excitable cells;

10

b) a pair of electro-stimulation electrodes positioned on the substrate within the at least one well and configured to electro-stimulate the excitable cells; and

15

c) at least a second pair of electrodes positioned within the at least one well and selected from the group consisting of a pair of impedance monitoring electrodes and extracellular recording electrode pair, wherein the pair of impedance monitoring electrodes are configured for monitoring cell-substrate impedance of cells attached to the substrate, and wherein the extracellular recording electrode pair are configured for monitoring extracellular potential of cells attached to the substrate.

20

21. The device according to claim 20, wherein a percentage of a surface area of the bottom of the at least one well occupied by the pair of electro-stimulation electrodes is selected from the group consisting of 5% or more, 10% or more, 20% or more, 30% or more, 50% or more, and 70% or more.

25

22. The device according to claim 20, wherein each of the electro-stimulation electrodes independently comprises an unbranched electrode structure or a branched electrode structure.

30

23. The device according to claim 20, wherein the at least second pair of electrodes is the pair of impedance measurement electrodes.

24. The device according to claim 23, wherein each electrode within the pair of impedance measurement electrodes comprises a plurality of electrode elements and is configured as one half of a pair of interdigitated electrodes.

5

25. The device according to claim 23, wherein each electrode of the pair of impedance monitoring electrodes has a same surface area.

10

26. The device according to claim 20, wherein the at least second pair of electrodes is the extracellular recording electrode pair.

27. The device according to claim 26, wherein the extracellular recording electrode pair comprising a recording electrode and reference electrode.

15

28. The device according to claim 27, wherein the recording electrode has a diameter from about 10 μm to about 200 μm or from about 30 μm to about 100 μm .

20

29. The device according to claim 27, wherein a ratio of the area of reference electrode to the area of recording electrode is selected from the group consisting of 2 or more, 10 or more, 100 or more, 1,000 or more, and 10,000 or more.

30. The device according to claim 27, wherein a reference electrode of the recording and reference electrode pair has an unbranched structure.

25

31. The device according to claim 20, wherein at least one electrode is shared between the pair of electro-stimulation electrodes and the at least second electrode pair.

32. The device according to claim 31, wherein the at least second electrode pair is the pair of impedance measurement electrodes.

30

33. The device according to claim 31, wherein the at least second electrode pair is the extracellular recording electrode pair.

34. The device according to claim 20, wherein the at least second electrode pair is the pair of impedance measurement electrodes, the device further comprising the extracellular recording pair as a third pair of electrodes.

5 35. The device according to claim 34, wherein at least one electrode is shared between the pair of impedance measurement electrodes and the recording and reference electrode pair.

36. A method for monitoring excitable cells, comprising:

- 10 a) providing the system according to claim 1;
- b) adding a sample of excitable cells to the device;
- 15 c) electro-stimulating the excitable cells; and
- d) monitoring electro-stimulated cells through the at least second pair of electrodes.

20 37. The method according to claim 36, wherein the excitable cells comprise cardiomyocytes or cardiomyocyte precursor cells.

25 38. The method according to claim 36, wherein the step of electro-stimulating the excitable cells is performed at a plurality of time intervals, optionally at regular time intervals.

30 39. The method according to claim 36, wherein the pair of impedance monitoring electrodes are communicatively coupled to the at least one analyzing module in the form of the impedance analyzer, and wherein the step of monitoring electro-stimulated cells comprises monitoring impedance of electro-stimulated cells.

40. The method according to claim 39, wherein impedance is monitored in millisecond time resolution.

41. The method according to claim 39, further comprising adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well.

42. The method according to claim 41, further comprising comparing an impedance-based parameter prior to and after adding the compound to identify whether a change occurs in the excitable cells in response to the compound.

43. The method according to claim 42, wherein the impedance-based parameter is compared between at least two different electro-stimulation intervals.

44. The method according to claim 42, wherein the impedance-based parameter is selected from the group consisting of an impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated from the cell index.

45. The method according to claim 43, wherein the impedance based parameter is plotted as an impedance-based curve over time and the step of comparing the impedance-based parameter between at least two different electro-stimulation intervals comprises comparing impedance-based curves between at least two different electro-stimulation intervals.

46. The method according to claim 38, wherein the excitable cells are added to each of at least two wells and the step of monitoring impedance is performed for each of the at least two wells, the method further comprising adding a compound suspected of affecting excitation contraction coupling of the excitable cells to a first of the at least two wells to form a test well and adding a control to a second of the at least two wells to form a control well, and comparing an impedance-based parameter between the test well and control well.

47. The method according to claim 46, wherein the impedance-based parameter is selected from the group consisting of the impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated
5 from the cell index.

48. The method according to claim 47, wherein the impedance-based parameter is plotted as an impedance-based curve over time and the step of comparing the impedance-based parameter between the test well and control well comprises
10 comparing the impedance-based curve of the test well to the impedance based curve of the control well.

49. The method according to claim 36, wherein the extracellular recording electrode pair is communicatively coupled to the at least one analyzing module in the
15 form of the extracellular recording amplifier, and wherein the step of monitoring electro-stimulated cells comprises extracellular recording of electro-stimulated cells.

50. The method according to claim 49, further comprising plotting extracellular potential from the extracellular recording of electro-stimulated cells over
20 time to form a field potential curve.

51. The method according to claim 49, where the step of extracellular recording is performed before and after the step of electro-stimulating the excitable cells.

25 52. The method according to claim 51, further comprising adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well.

53. The method according to claim 52, further comprising comparing
30 extracellular potential of the excitable cells prior to and after adding the compound to identify changes in extracellular potential in response to the compound.

54. The method according to claim 46, wherein the excitable cells are added to each of at least two wells and the step of extracellular recording is performed for each of the at least two wells, the method further comprising adding a compound suspected of affecting excitation contraction coupling of the excitable cells to a first of the at least two wells to form a test well and adding a control to a second of the at least two wells to form a control well, and comparing extracellular potential between the test well and control well.

55. The method according to claim 54, wherein the extracellular recording measurements are performed before and after adding the compound.

56. The method according to claim 36, wherein the pair of impedance monitoring electrodes are communicatively coupled to the impedance analyzer and the extracellular recording electrode pair is communicatively coupled to the extracellular recording amplifier, further wherein the step of monitoring electro-stimulated cells comprises impedance monitoring and extracellular recording of electro-stimulated cells.

57. The method according to claim 56, wherein at least one electrode is shared between two pairs of electrodes selected from the group consisting of the pair of electro-stimulation electrodes and the pair of impedance monitoring electrodes, the pair of electro-stimulation electrodes and the extracellular recording electrode pair, and the pair of impedance monitoring electrodes and the extracellular recording electrode pair, further wherein the sharing of at least the electrode is performed by switching communication to the at least one electrode.

58. The method according to claim 56, further comprising adding a compound suspected of affecting excitation contraction coupling of the excitable cells to the at least one well.

59. The method according to claim 58, further comprising comparing an impedance-based parameter and extracellular field potential prior to and after adding the

compound to identify whether a change occurs in the excitable cells in response to the compound.

5 60. The method according to claim 59, wherein the impedance-based parameter is selected from the group consisting of an impedance measurement, a cell index calculated from the impedance measurement, and a cell change index calculated from the cell index.

10 61. The method according to claim 59, wherein the impedance based parameter is plotted as an impedance-based curve over time and the extracellular field potential is plotted as field potential over time and the step of comparing the impedance-based parameter and extracellular field potential between at least two different electro-stimulation intervals comprises comparing impedance-based curves and comparing field potential curves between at least two different electro-stimulation intervals.

15

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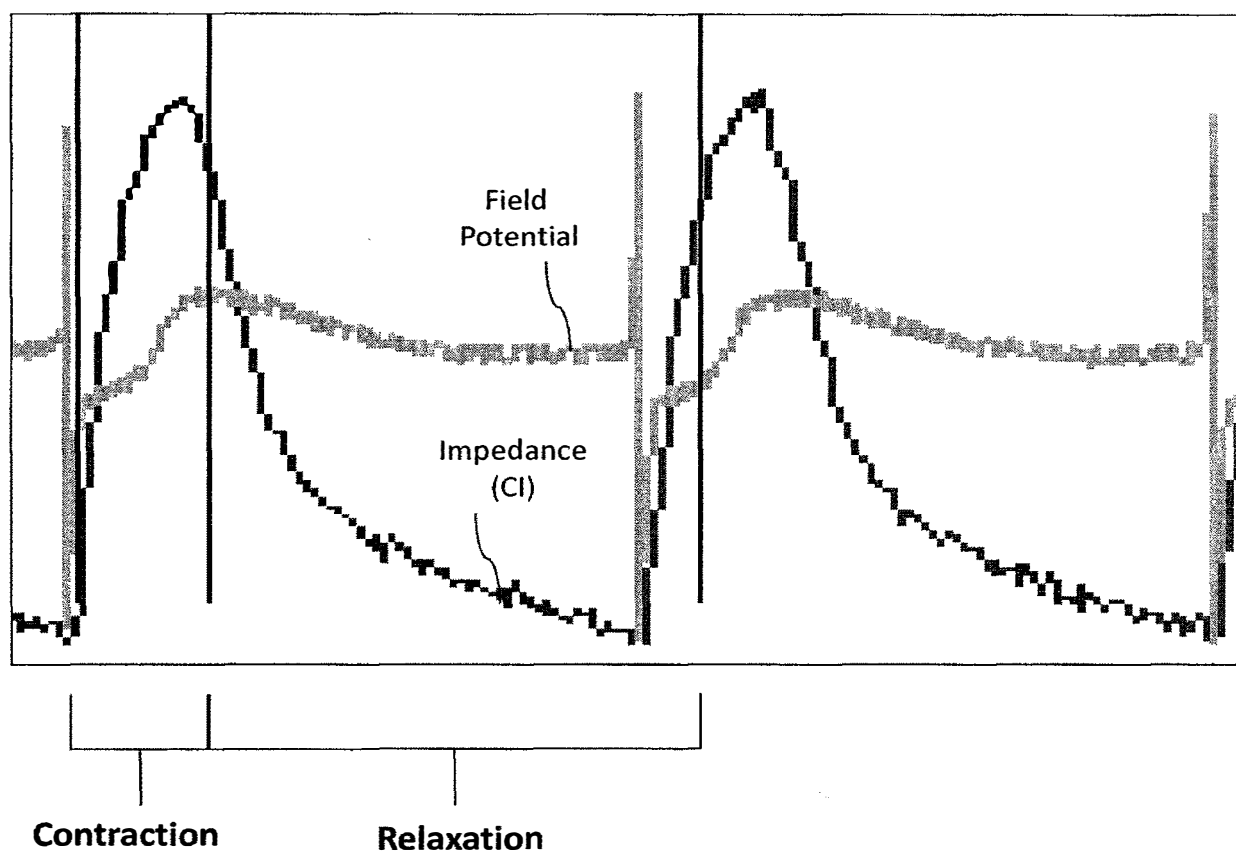


FIG. 1

10A

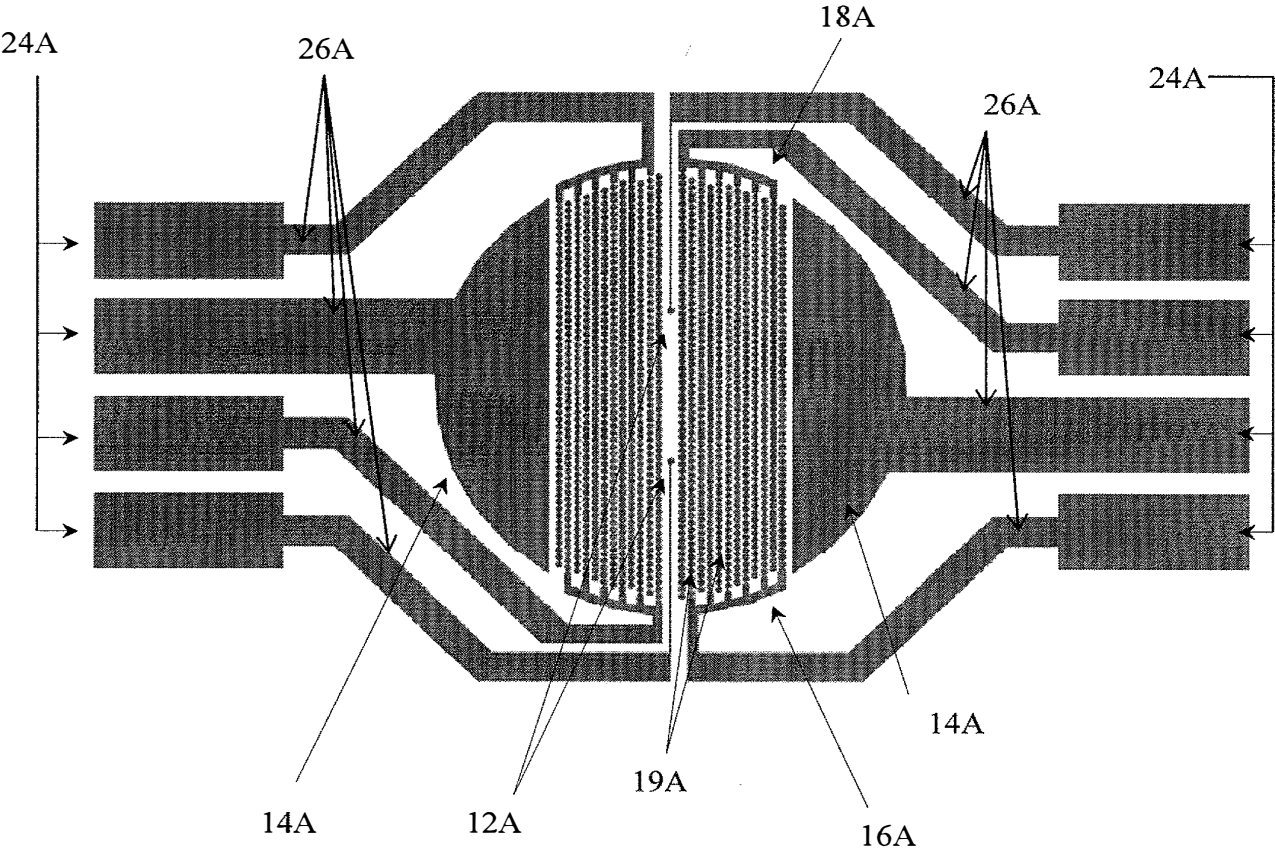


FIG. 2A

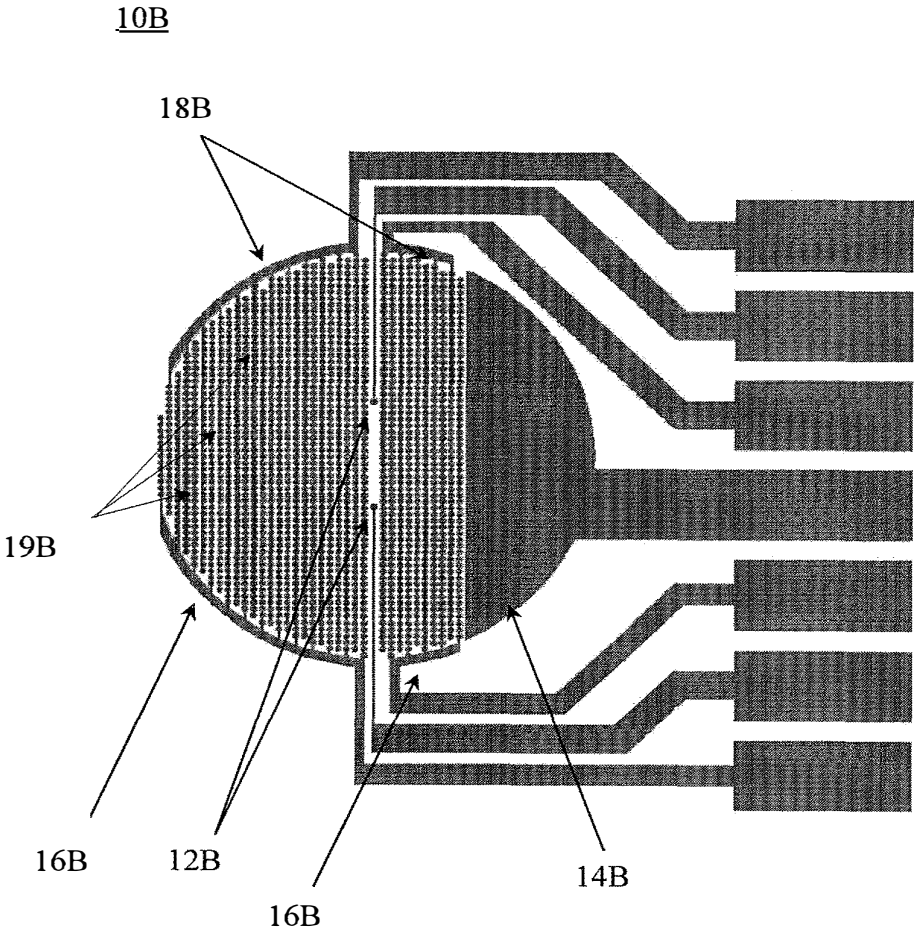


FIG. 2B

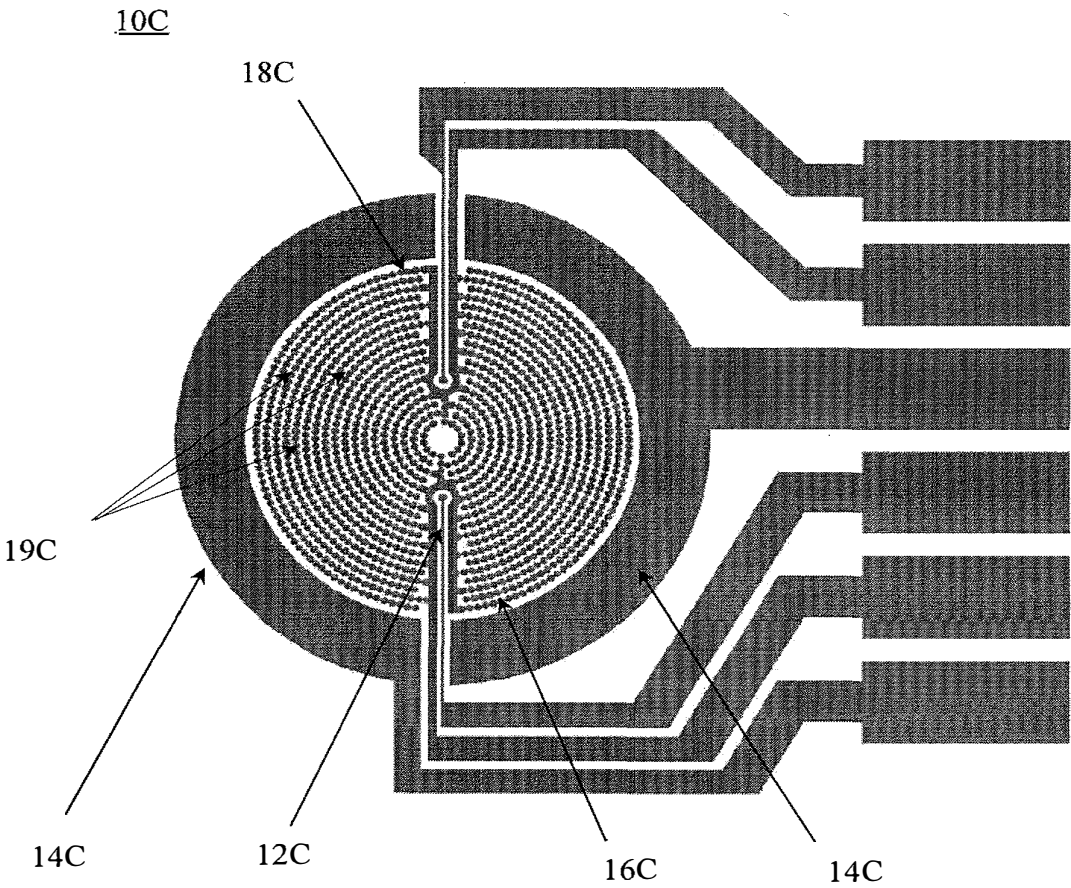


FIG. 2C

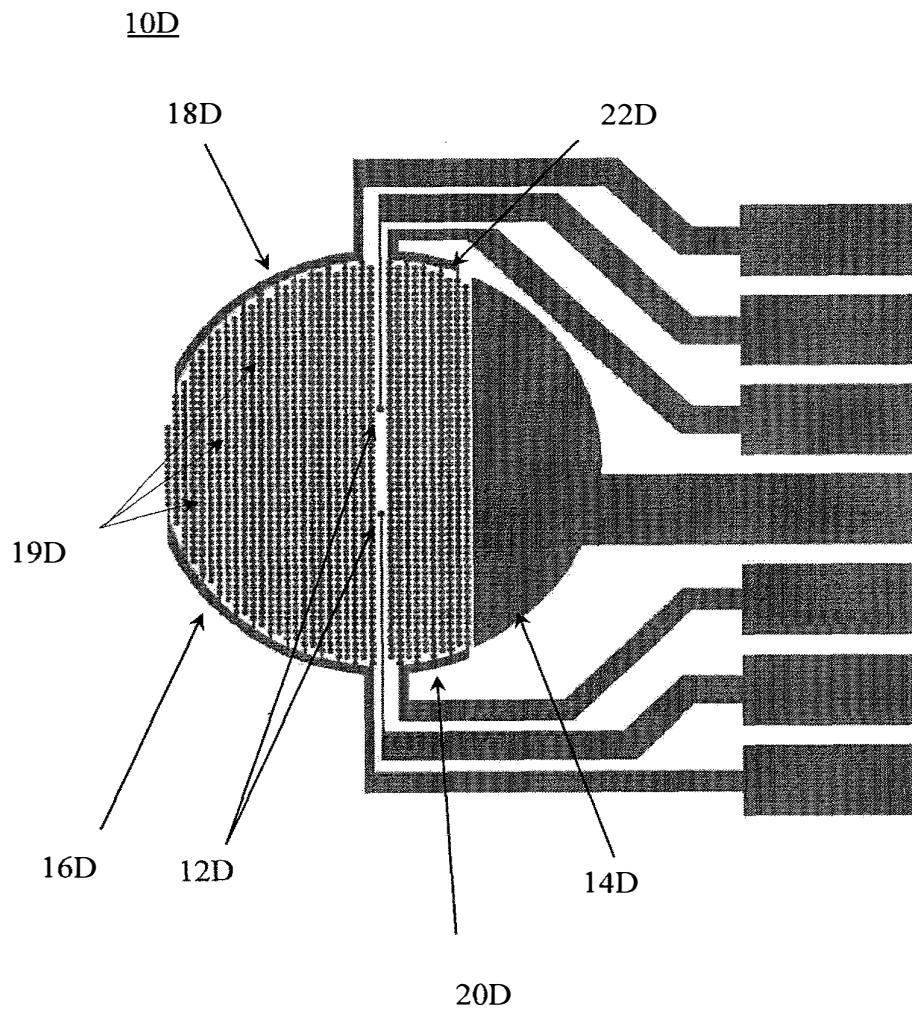


FIG. 2D

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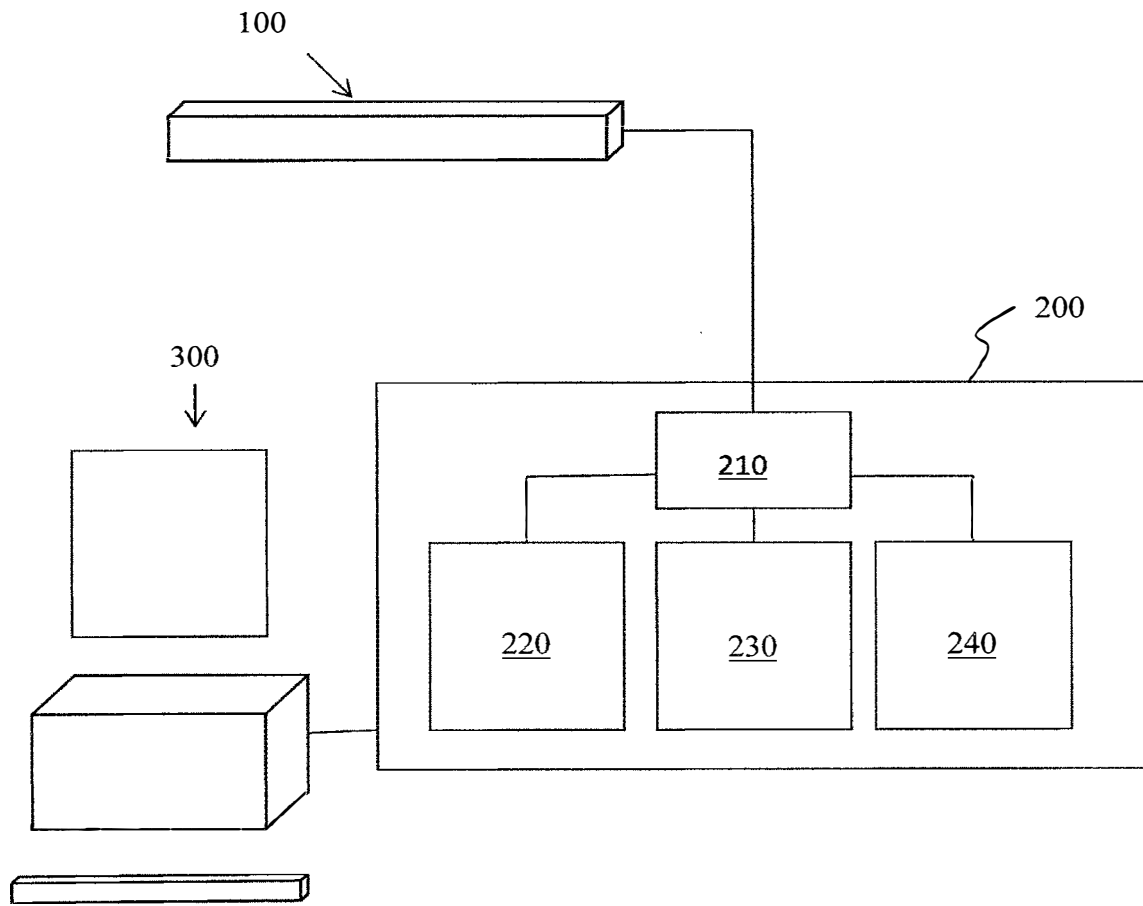


FIG. 3

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File System Help

1110291443CD.ppt (D:1110291443CD)

File Edit Setup Steps Database

Exp Notes Layout Schedule Cell Index Well Graph Plot Data Analysis Message Audit Trail

User: USER Browse

Data Directory: F:\Wayne\Data and analysis\Test HL-1 cells\2nd test

Experiment Name: HL-1 stimulation experiment

Device Part No:

Experiment Purpose: Test the combination of electric stimulation and compound treatment in HL-1 atria cells.

Experiment Procedures:

Experiment Conclusion:

System Information

Software Version:	1.0.0.1104
Analyzer Model:	Cardio
Analyzer SN:	391110110399
FW Version:	V4.0
Station Model:	W777
Station SN:	
Station FW:	V4.0
Self Test Val:	100.0500

FIG. 4A

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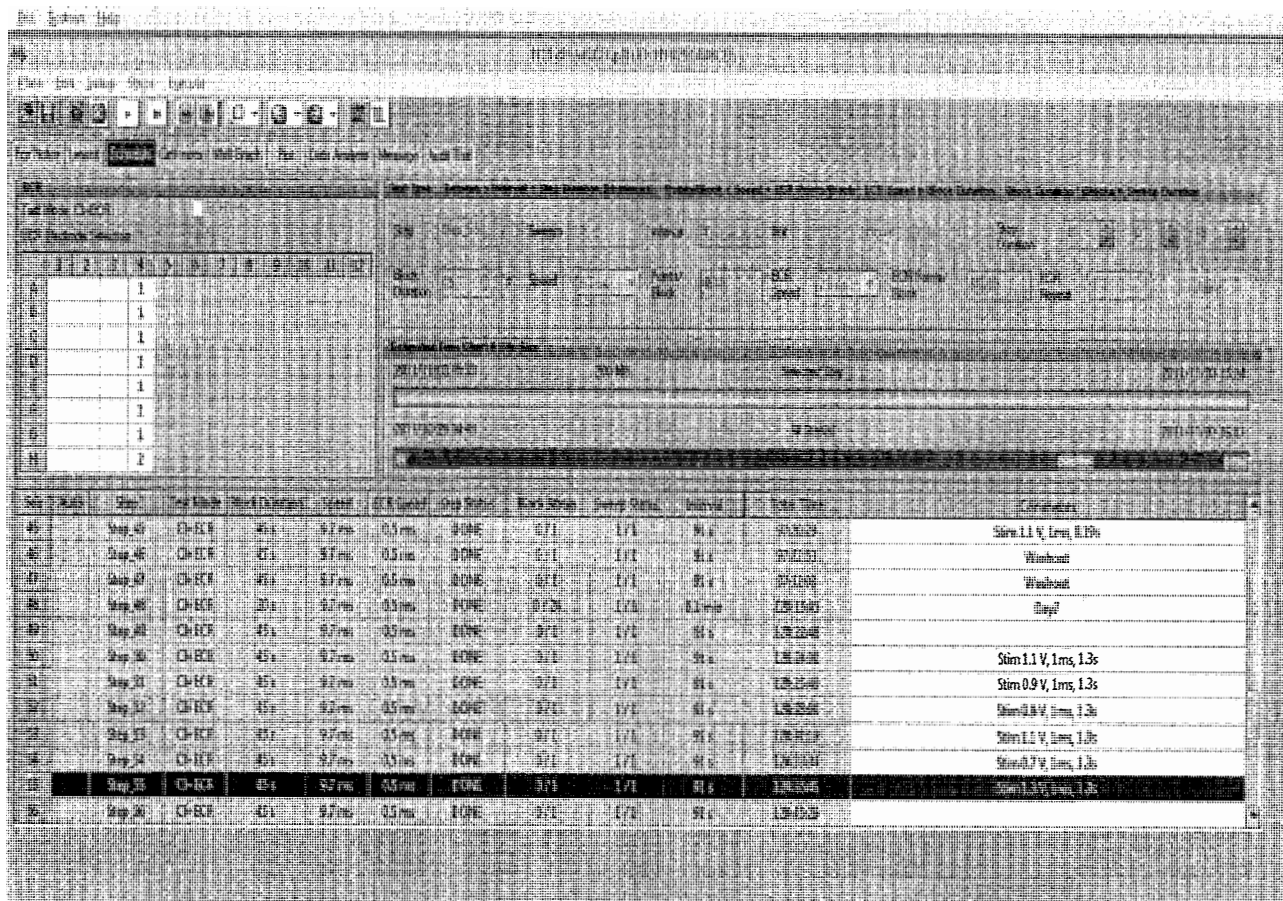


FIG. 4C

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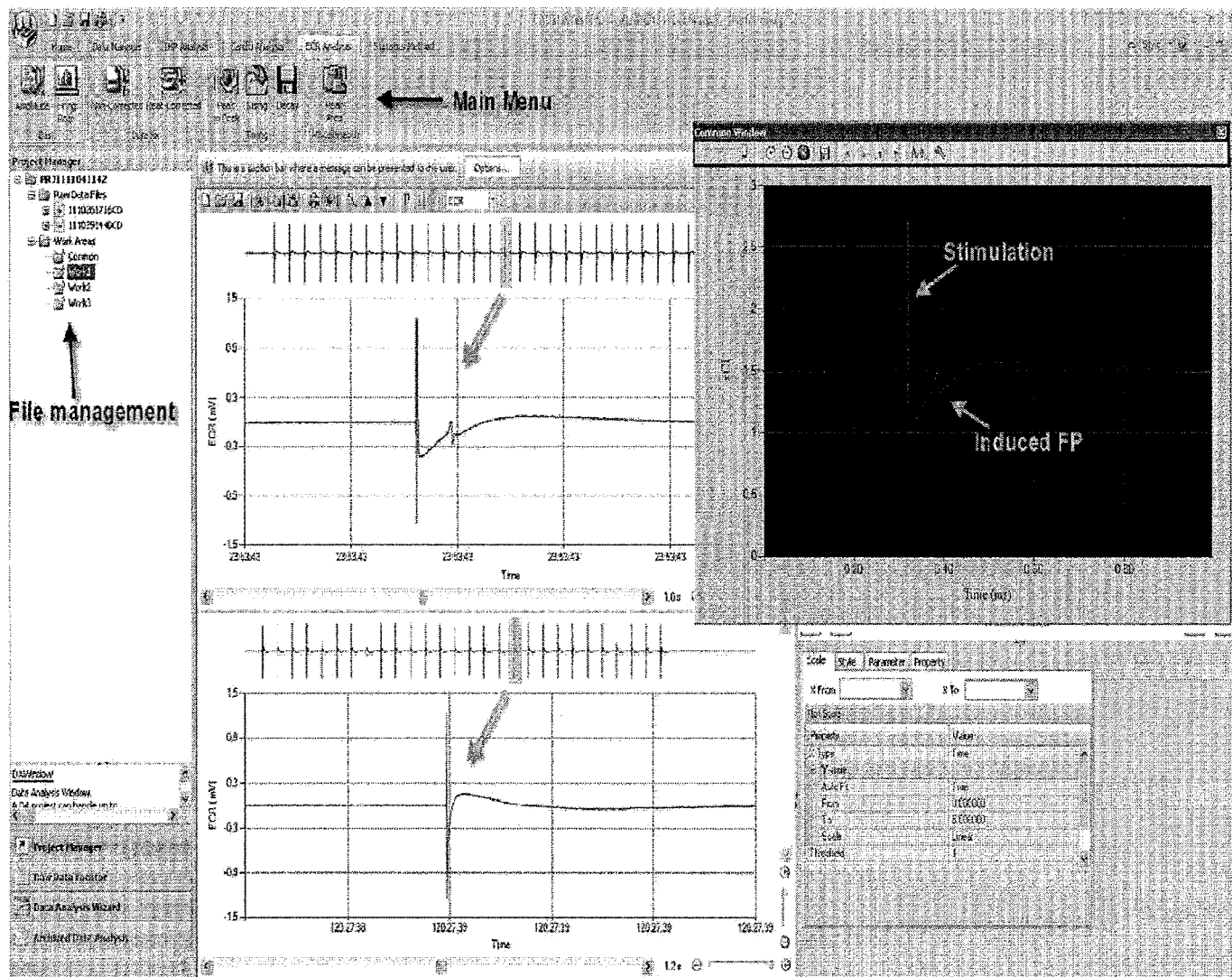


FIG. 4D

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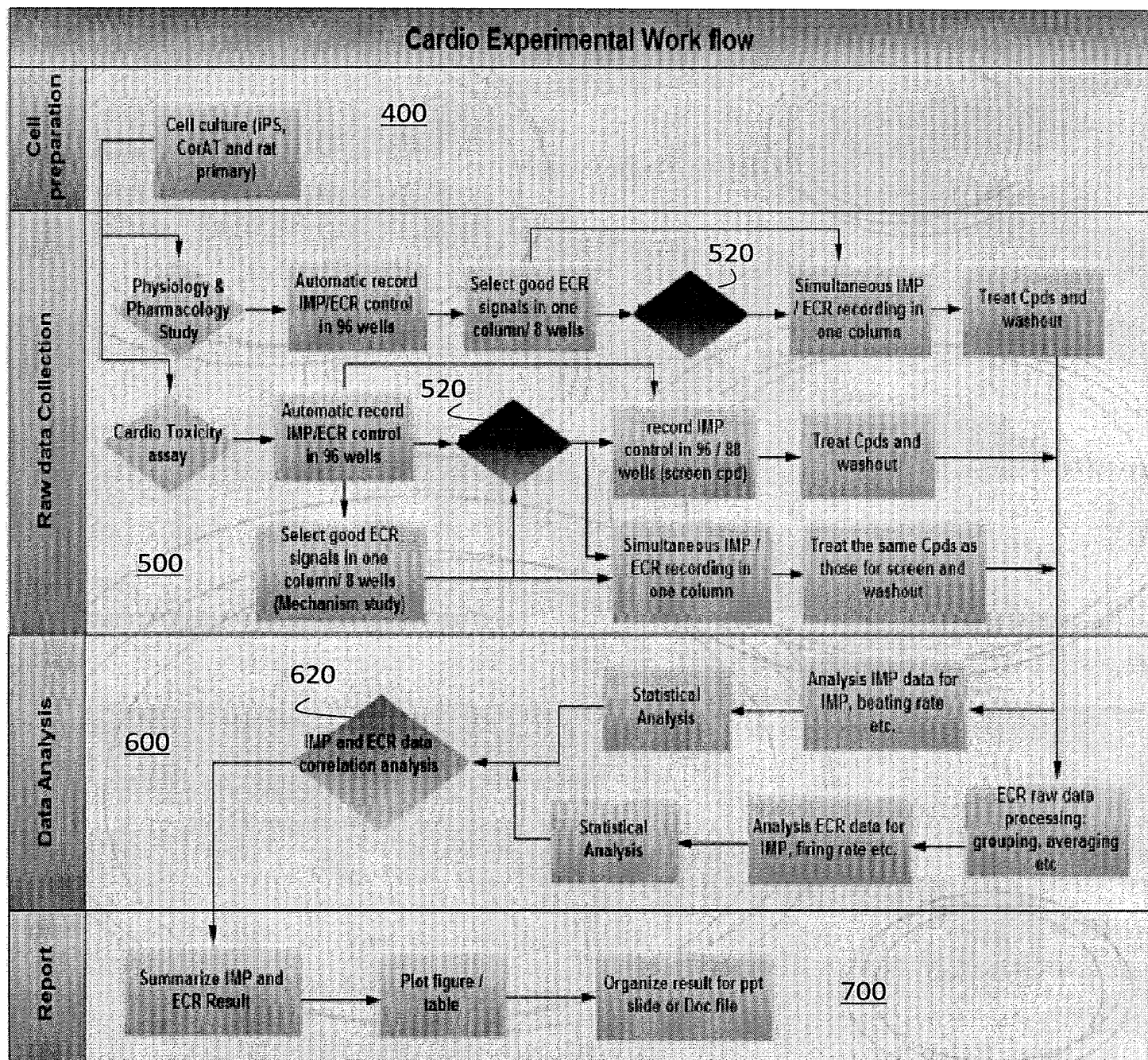


FIG. 5

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FIG. 6A

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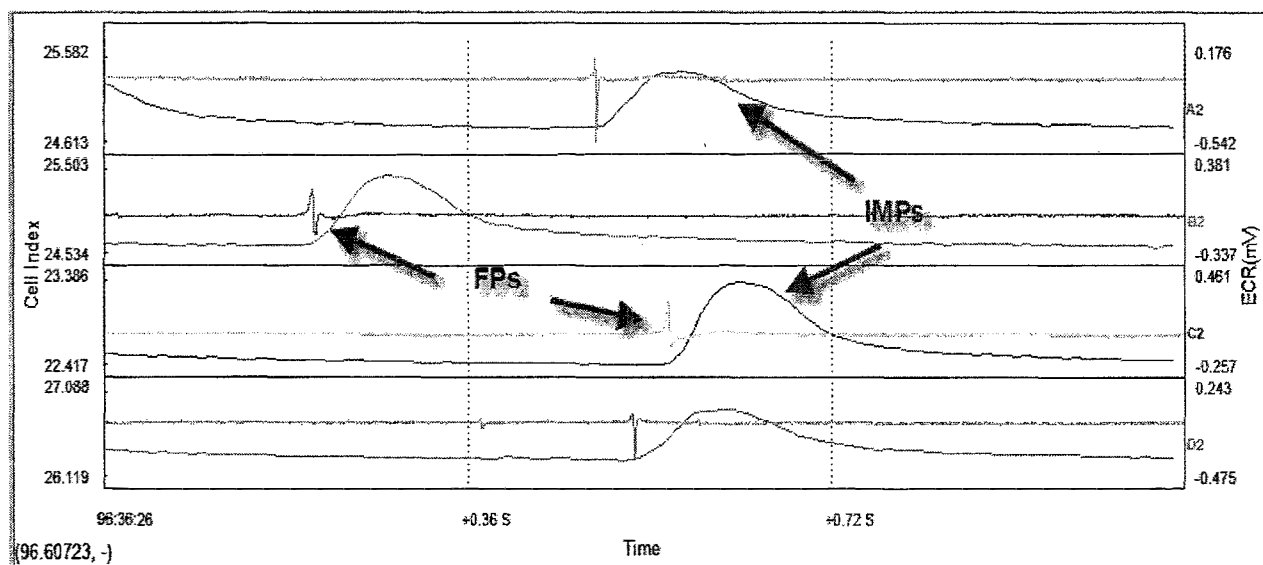


FIG. 6B

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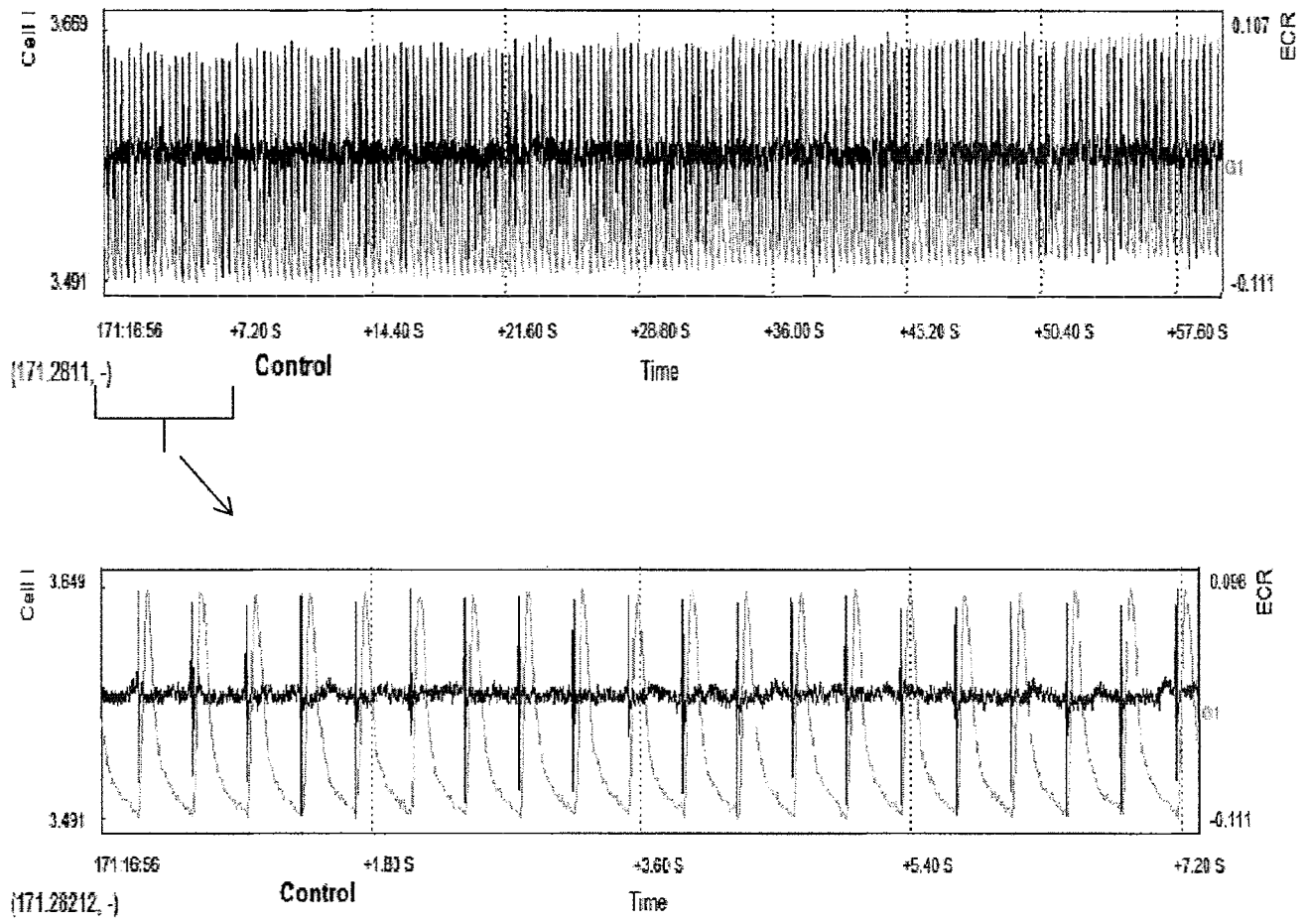


FIG. 7A

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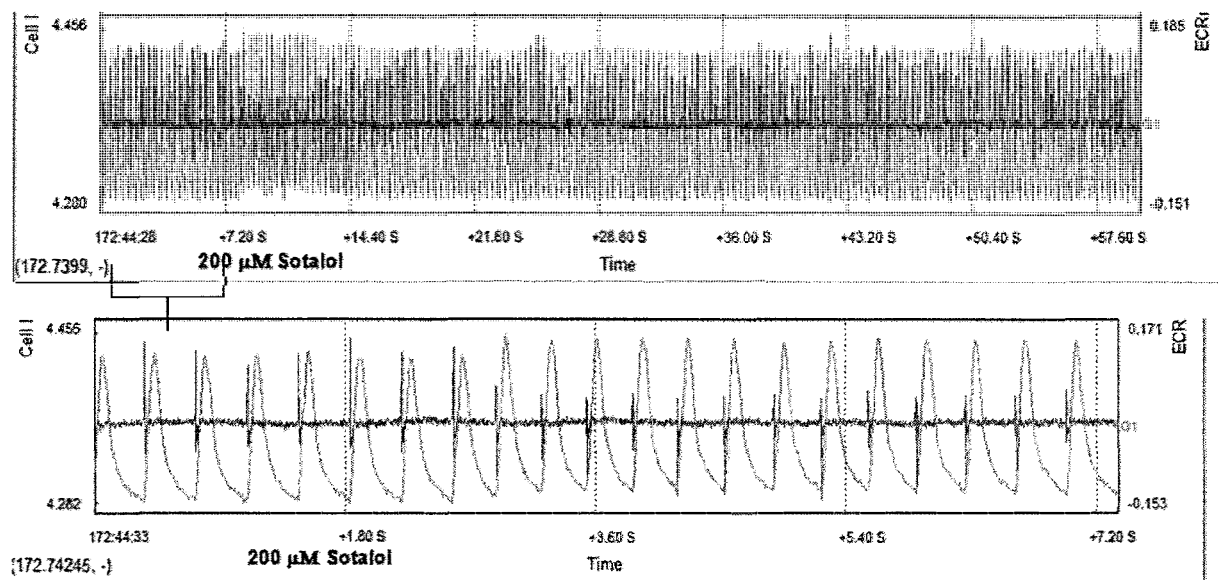


FIG. 7B

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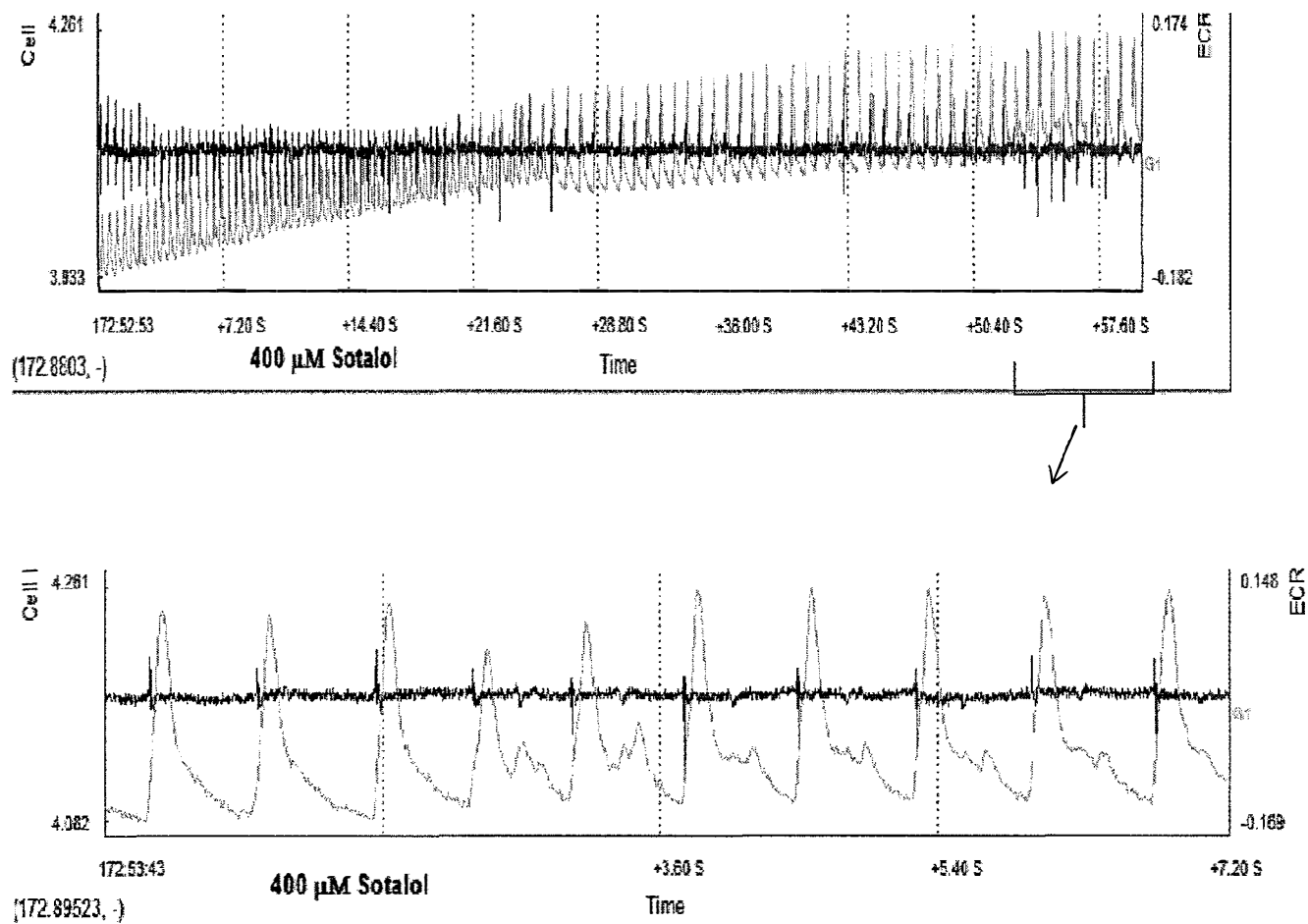


FIG. 7C

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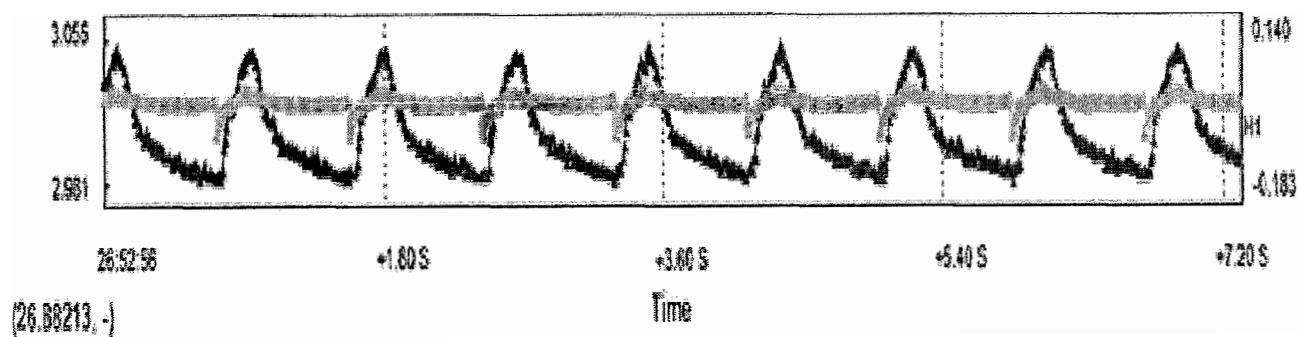


FIG. 8A

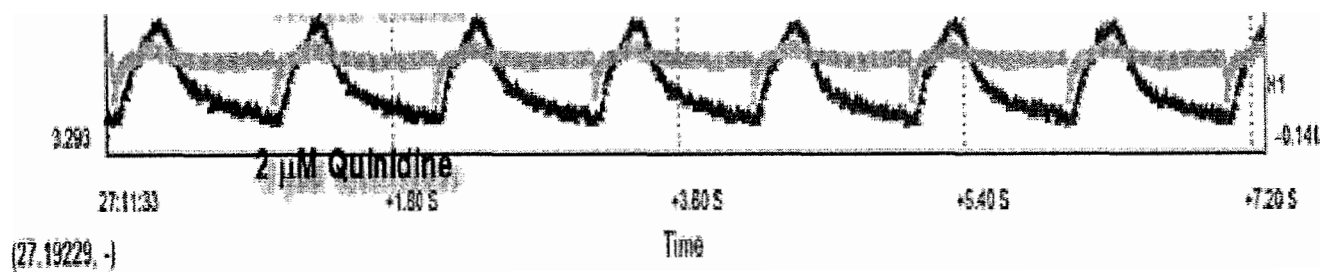


FIG. 8B

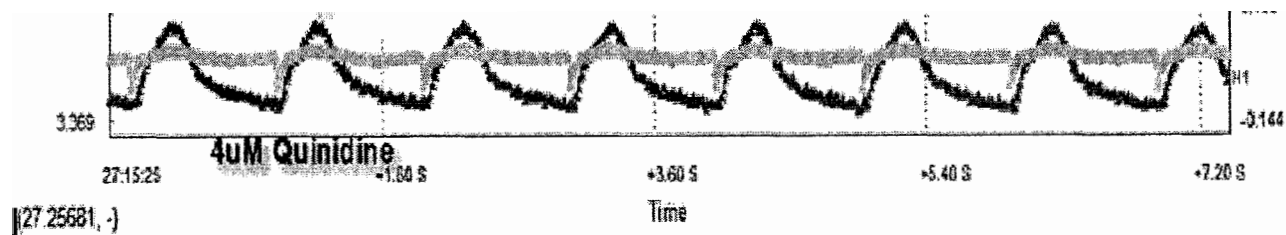


FIG. 8C

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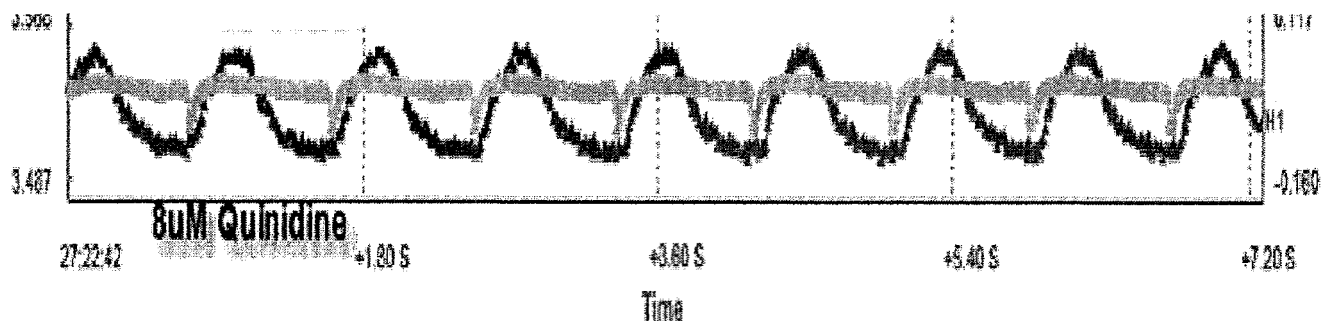


FIG. 8D

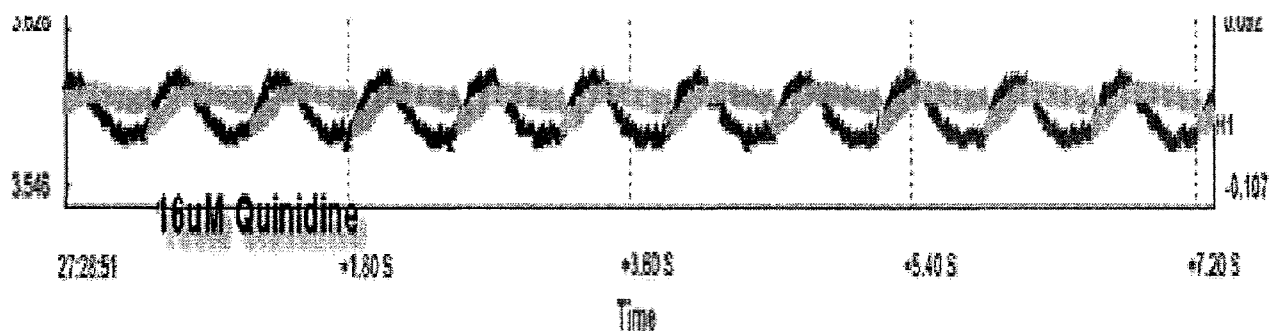
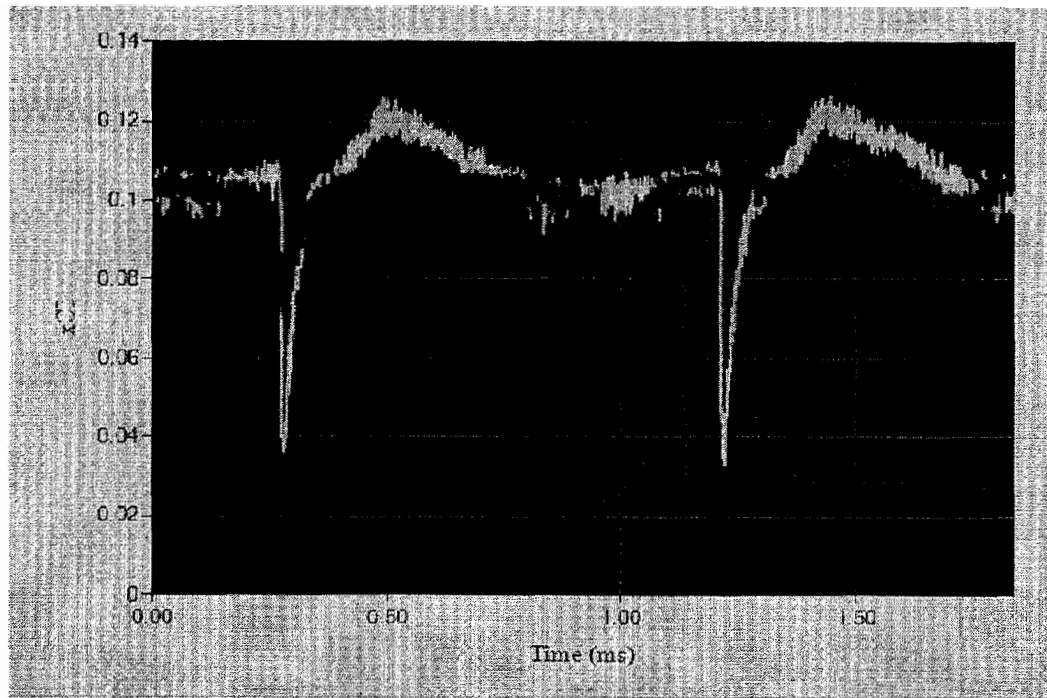
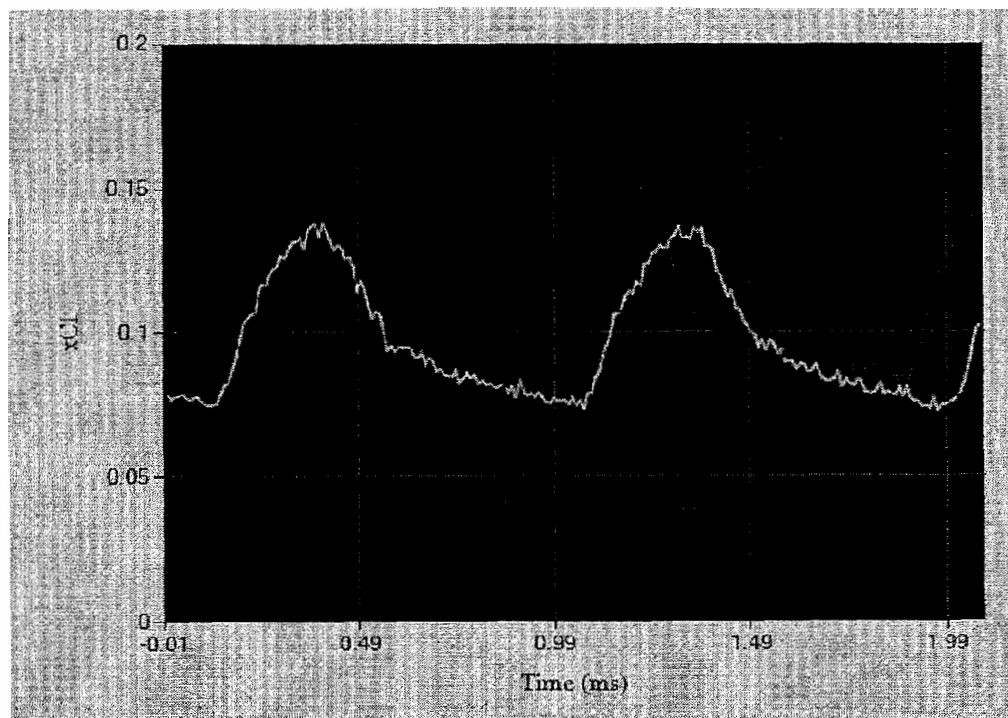


FIG. 8E

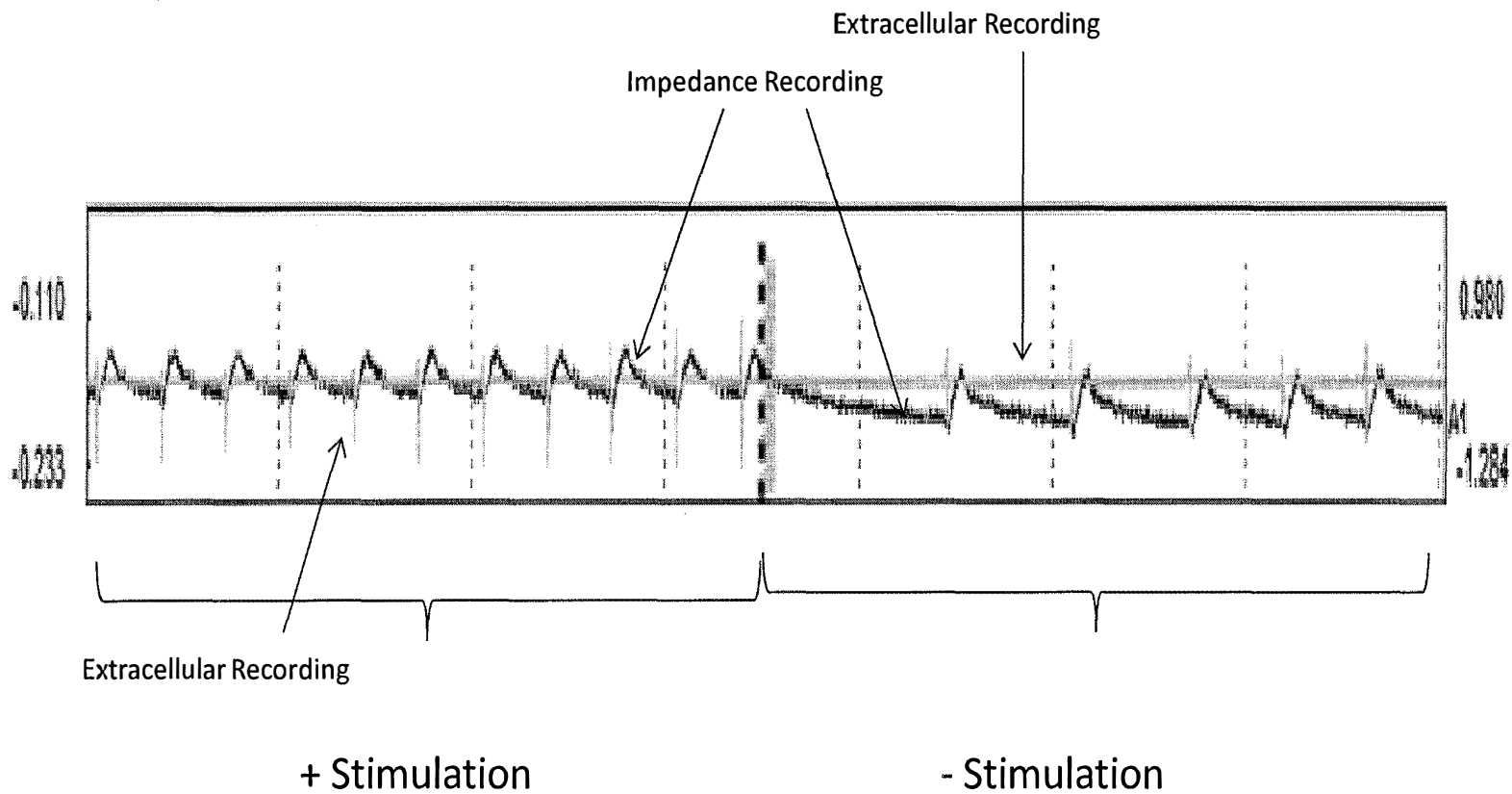
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**FIG. 6F****FIG. 8G**

Field Stimulation of Cor.AT Cells Using the Cardio-ECR System

WO 2014/085727

PCT/US2013/072439

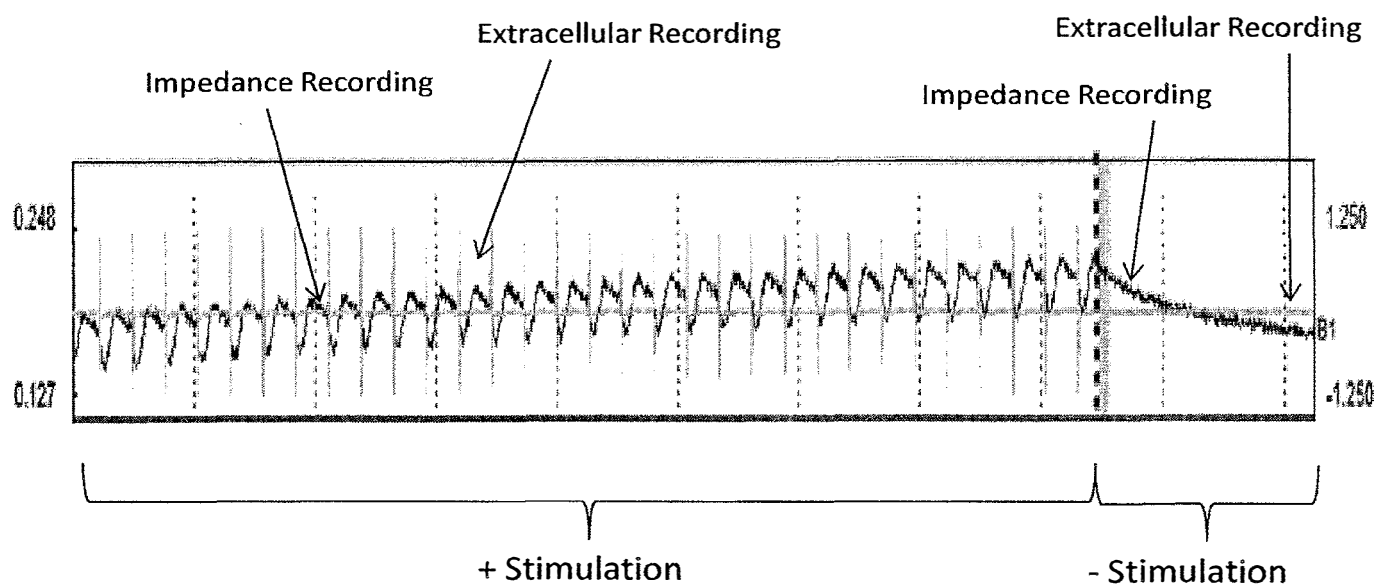


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FIG. 9

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Field Stimulation of Rat Neonatal Cardiomyocytes Using the Cardio-ECR System

**FIG. 10**

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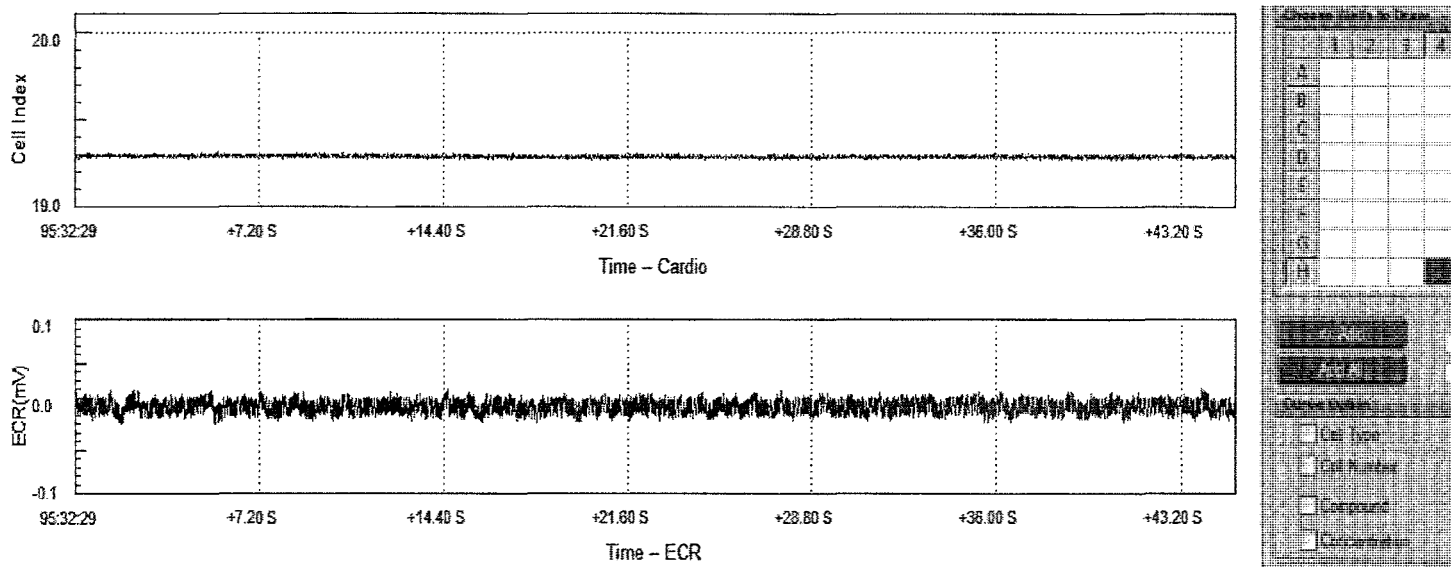


FIG. 11A

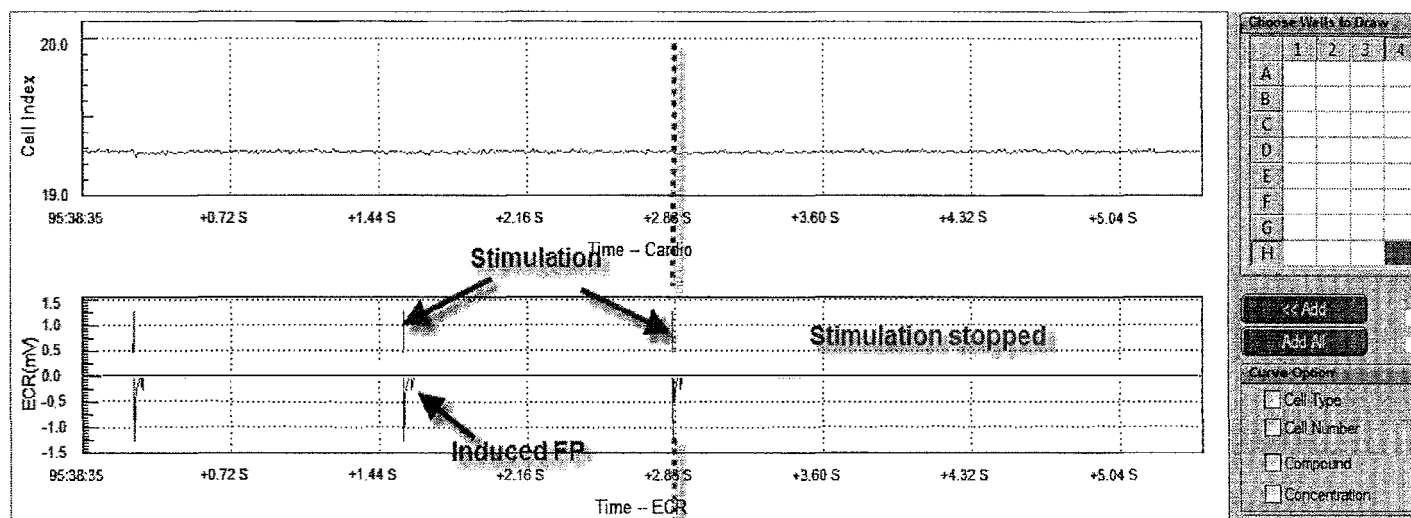


FIG. 11B

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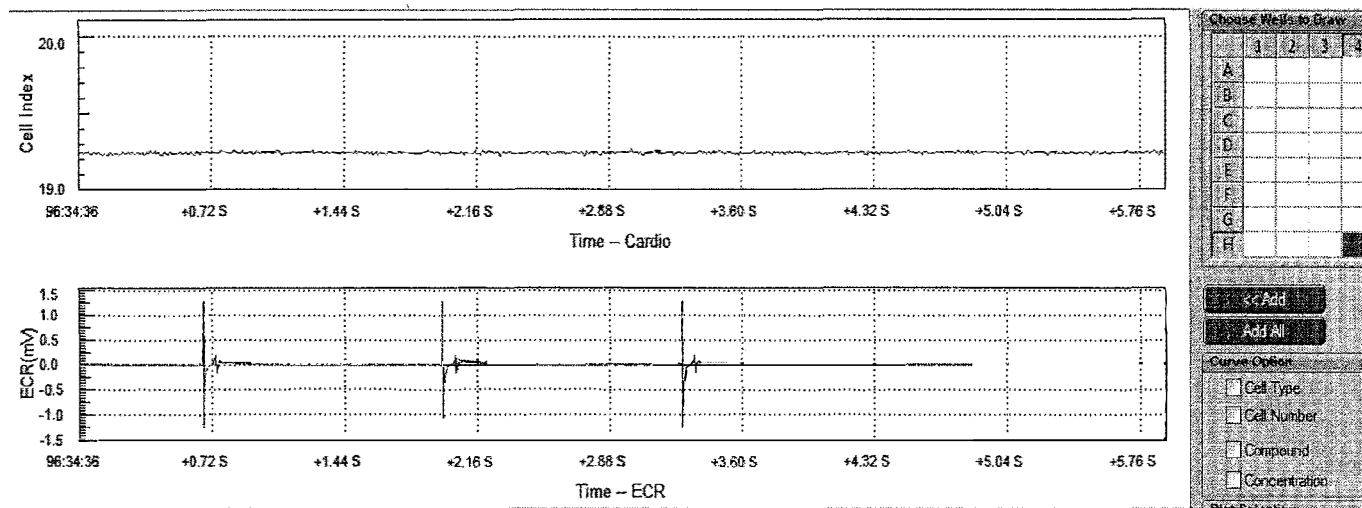


FIG. 11C

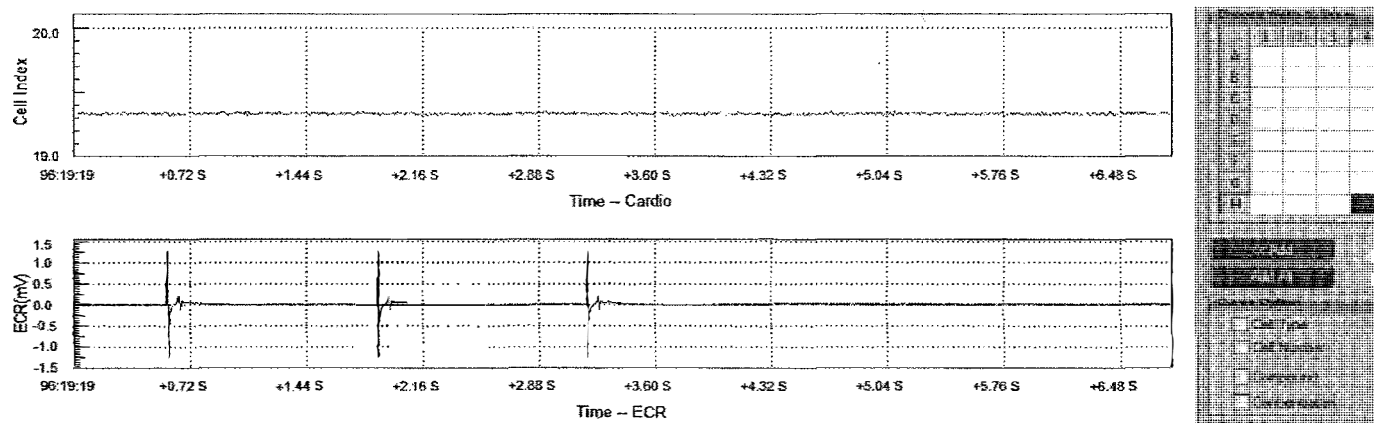


FIG. 11D

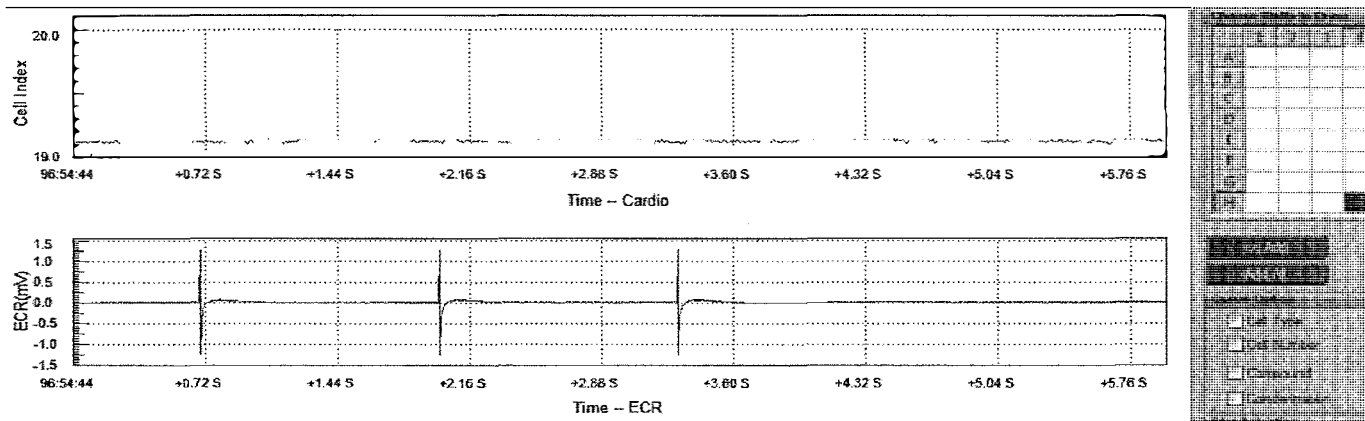


FIG. 11E

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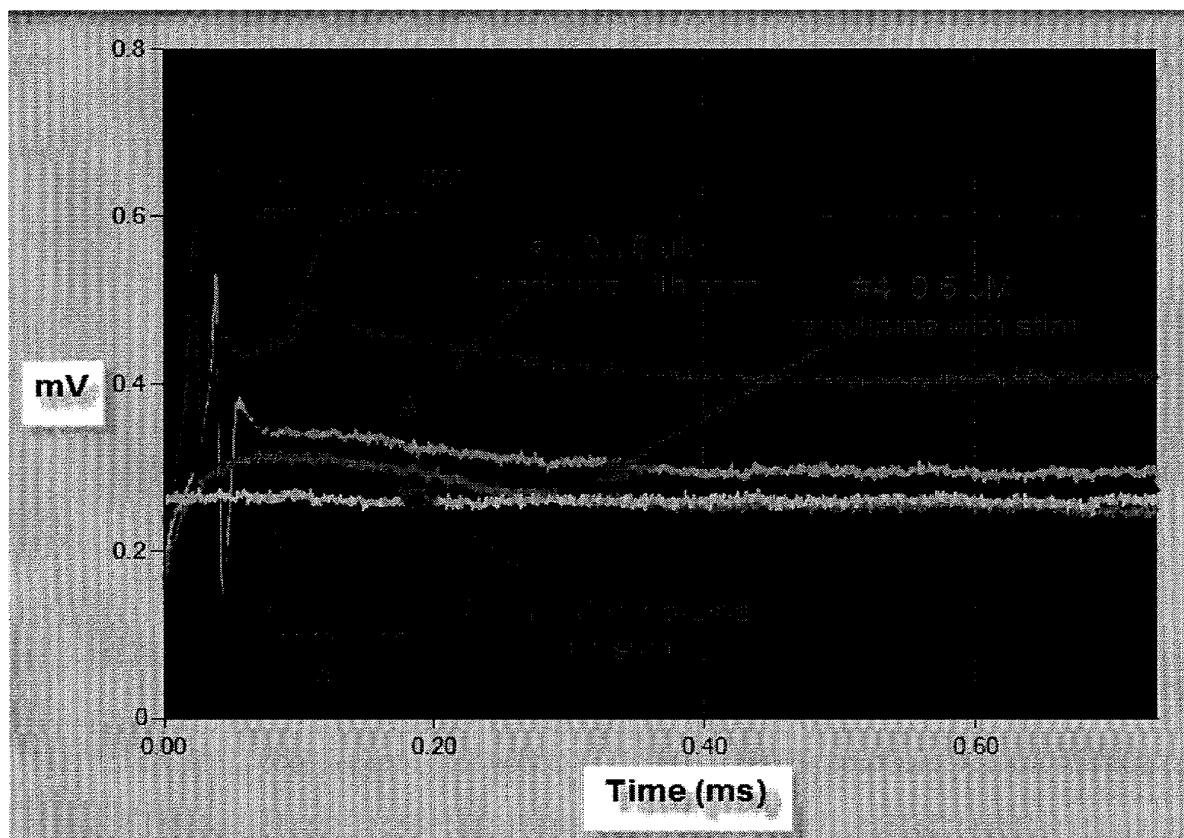


FIG. 11F

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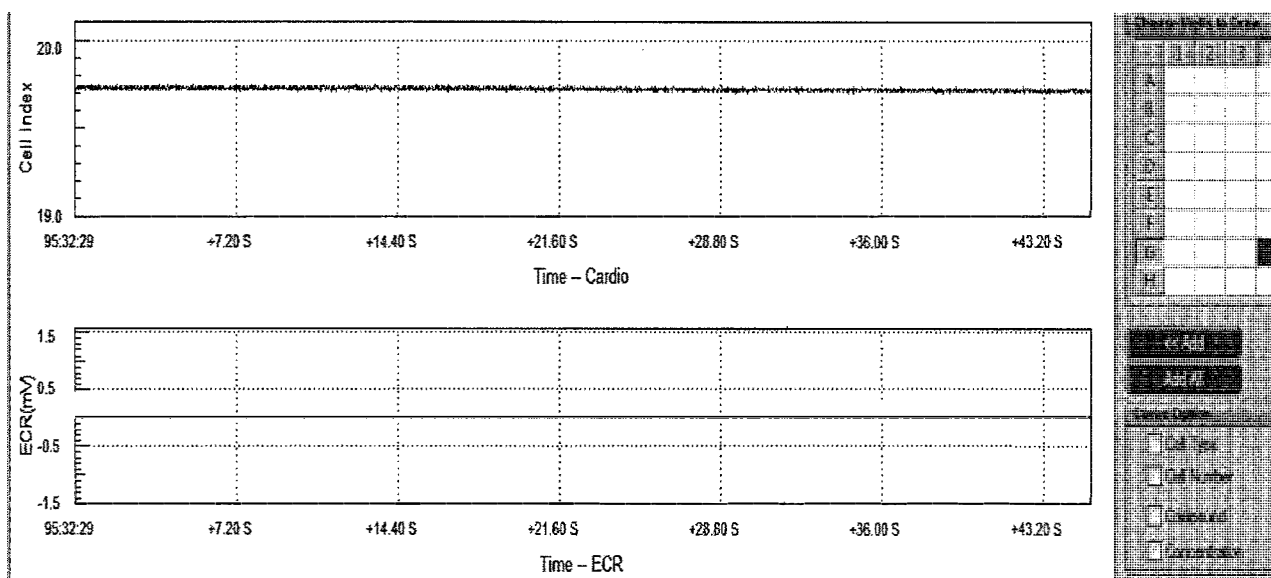


FIG. 12A

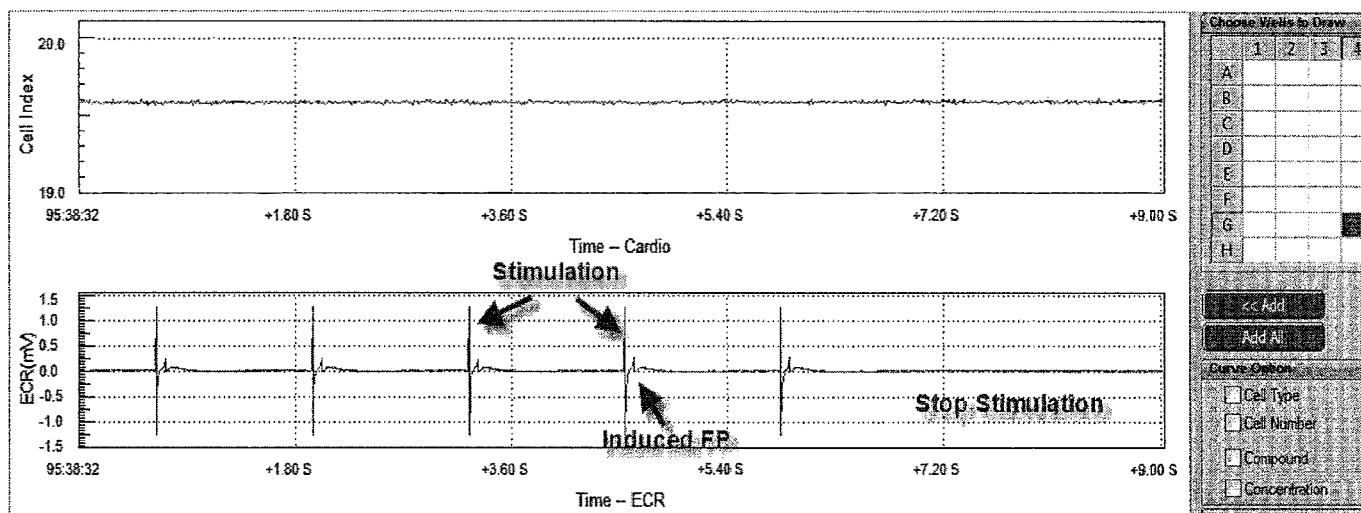


FIG. 12B

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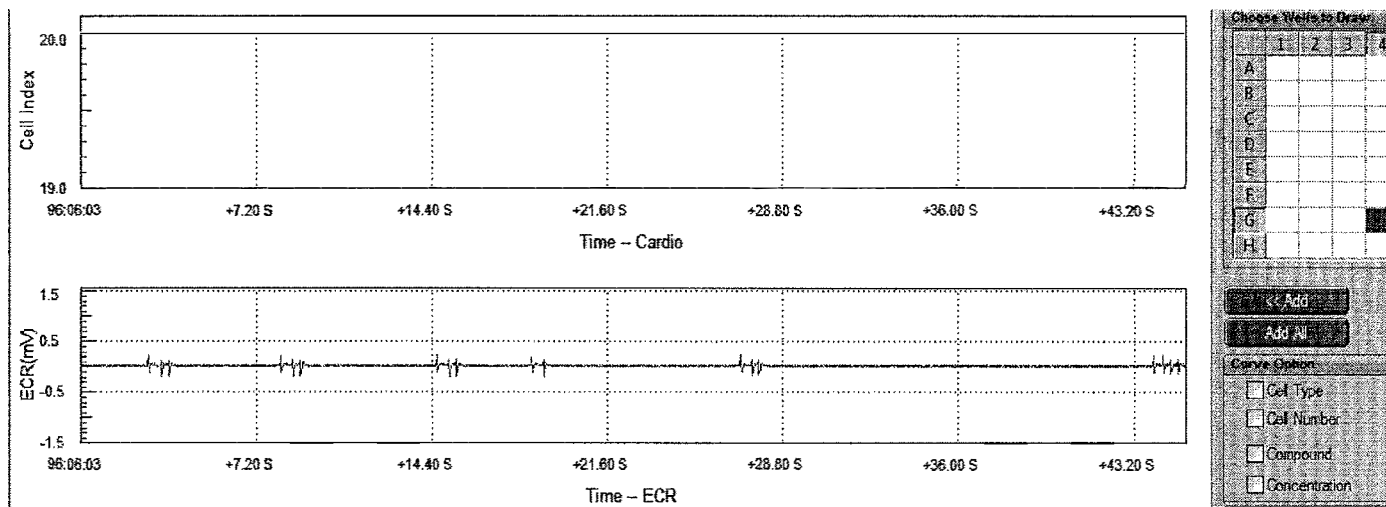


FIG. 12C

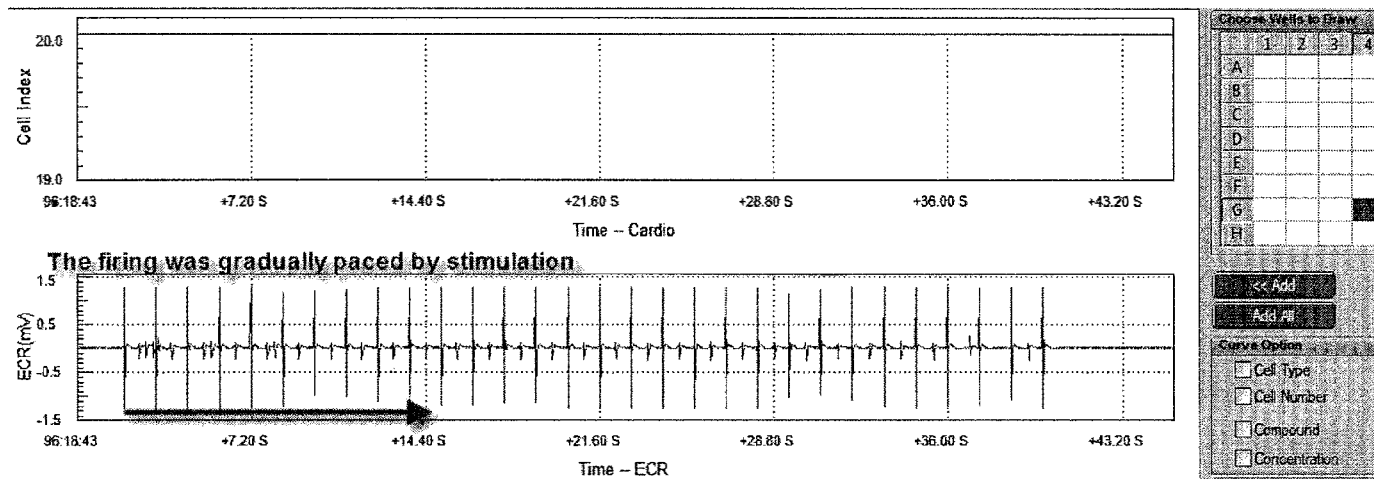


FIG. 12D

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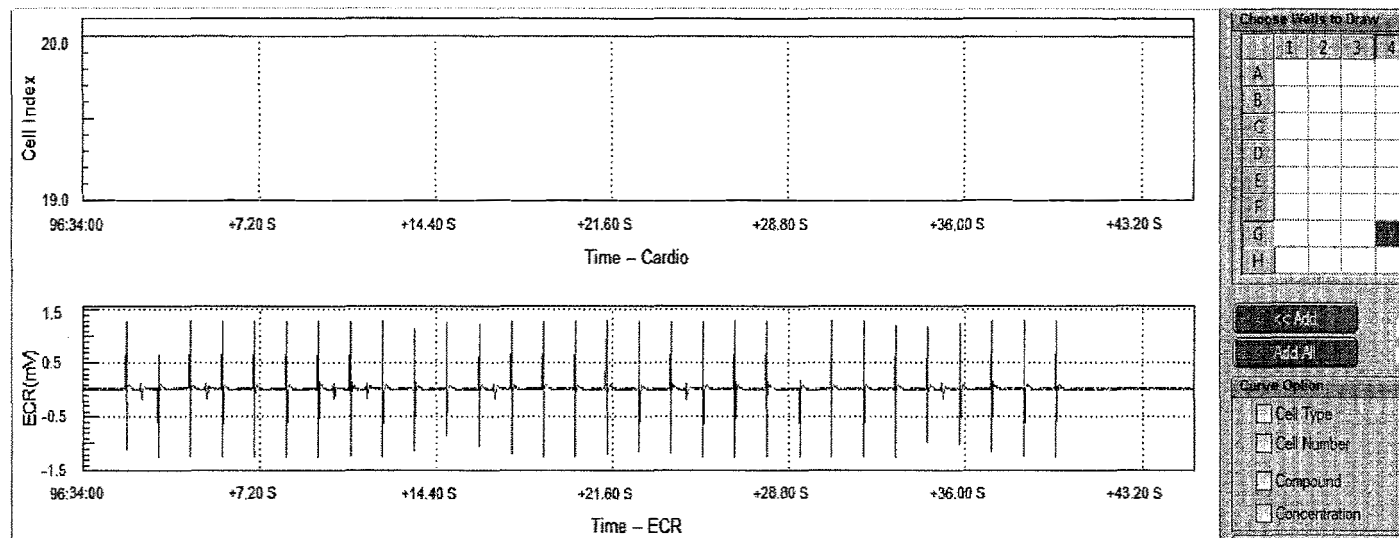


FIG. 12E

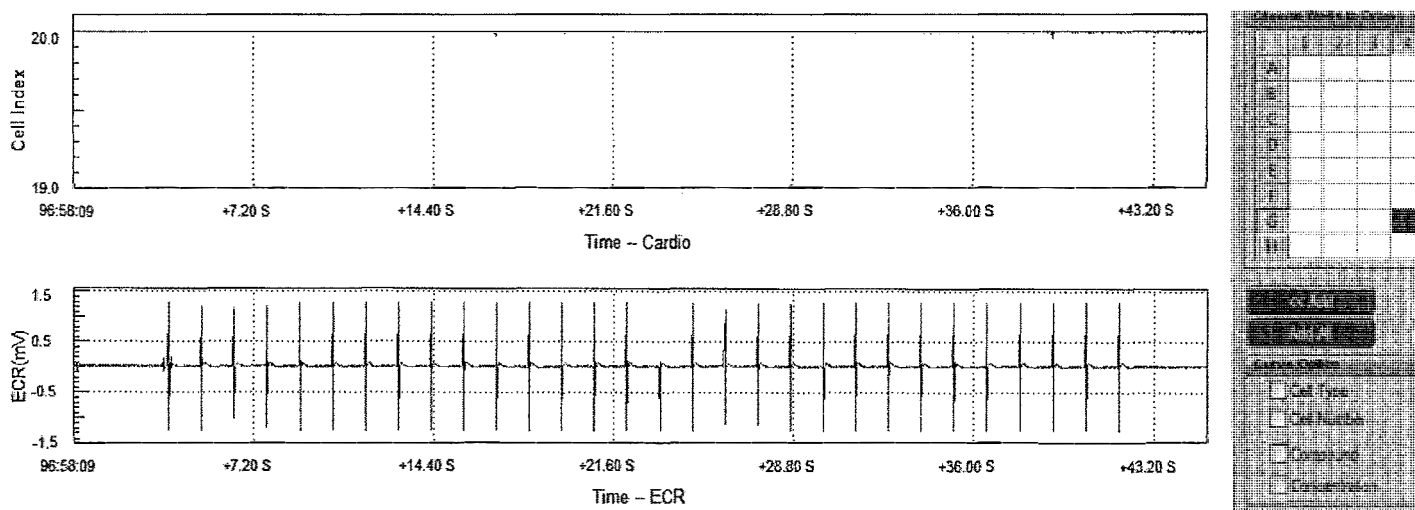


FIG. 12F

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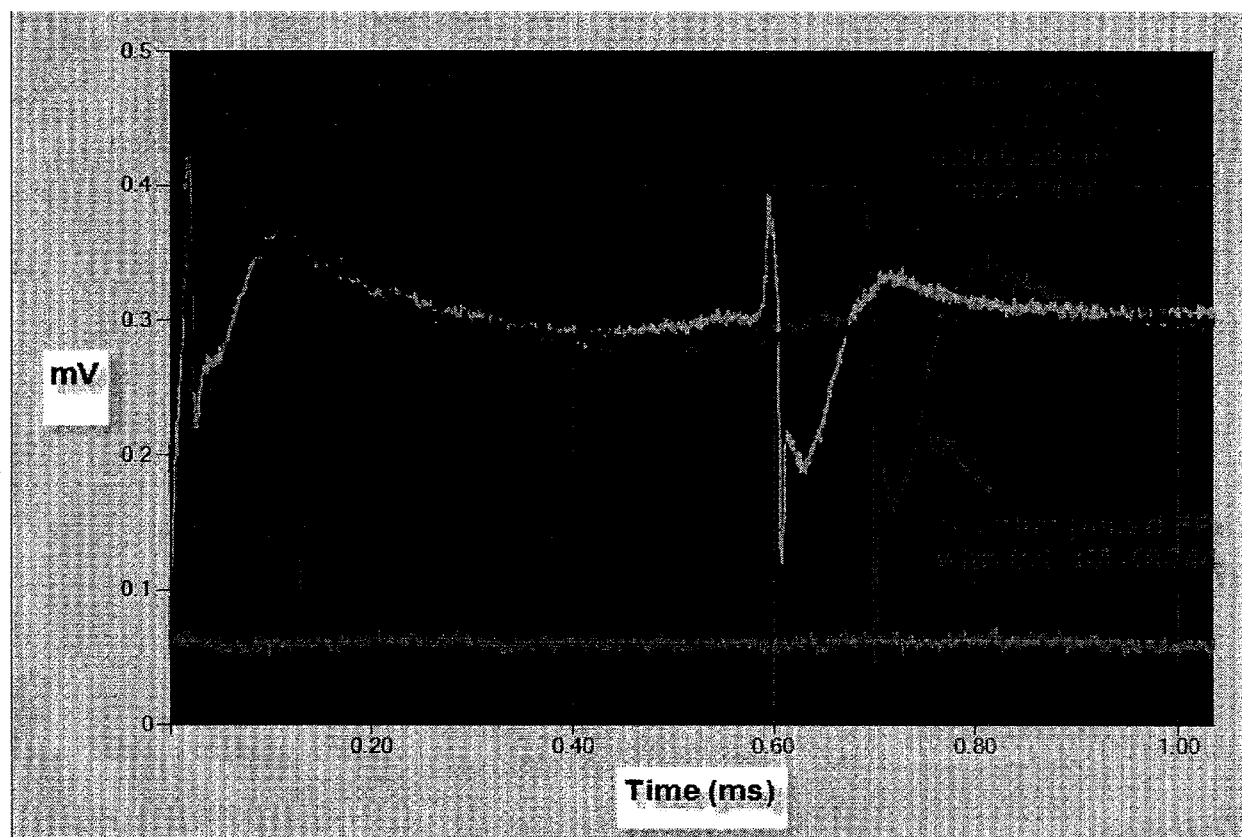


FIG. 12G

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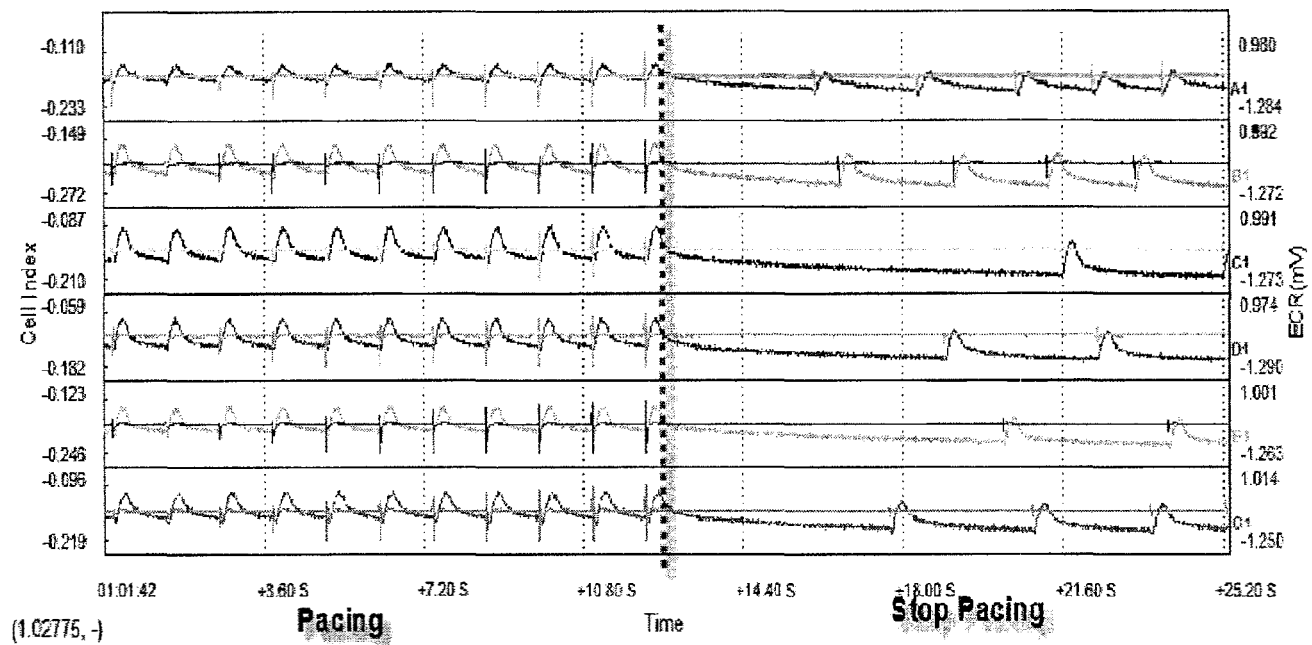


FIG. 13A

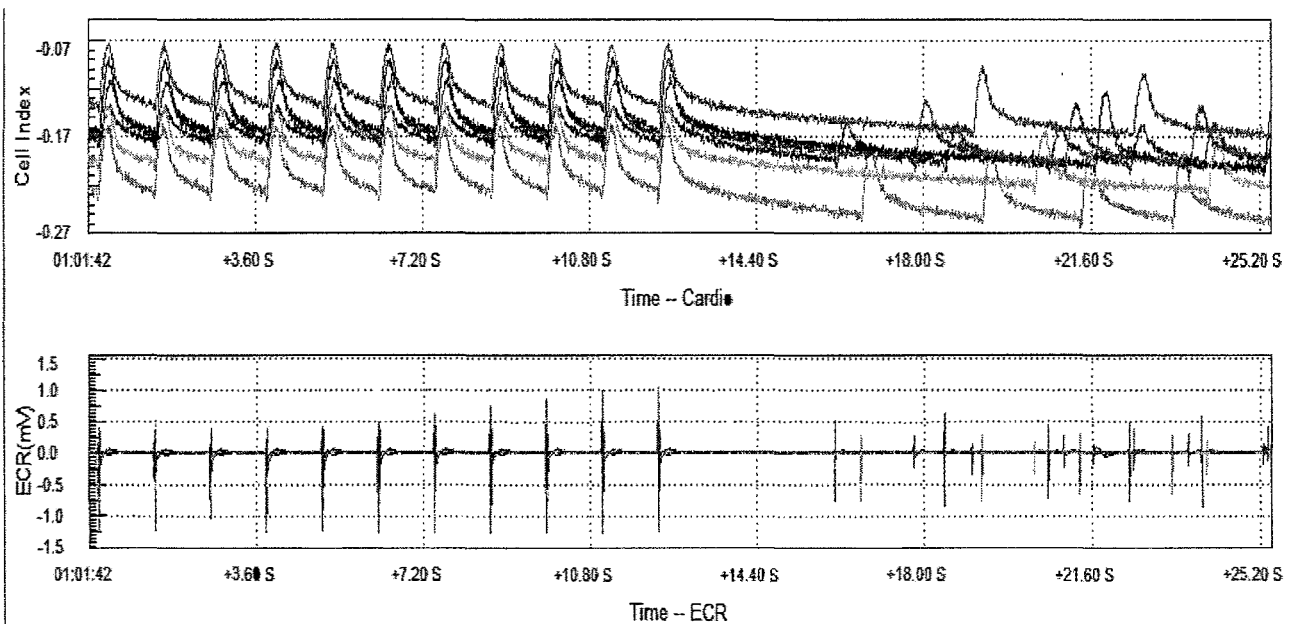


FIG. 13B

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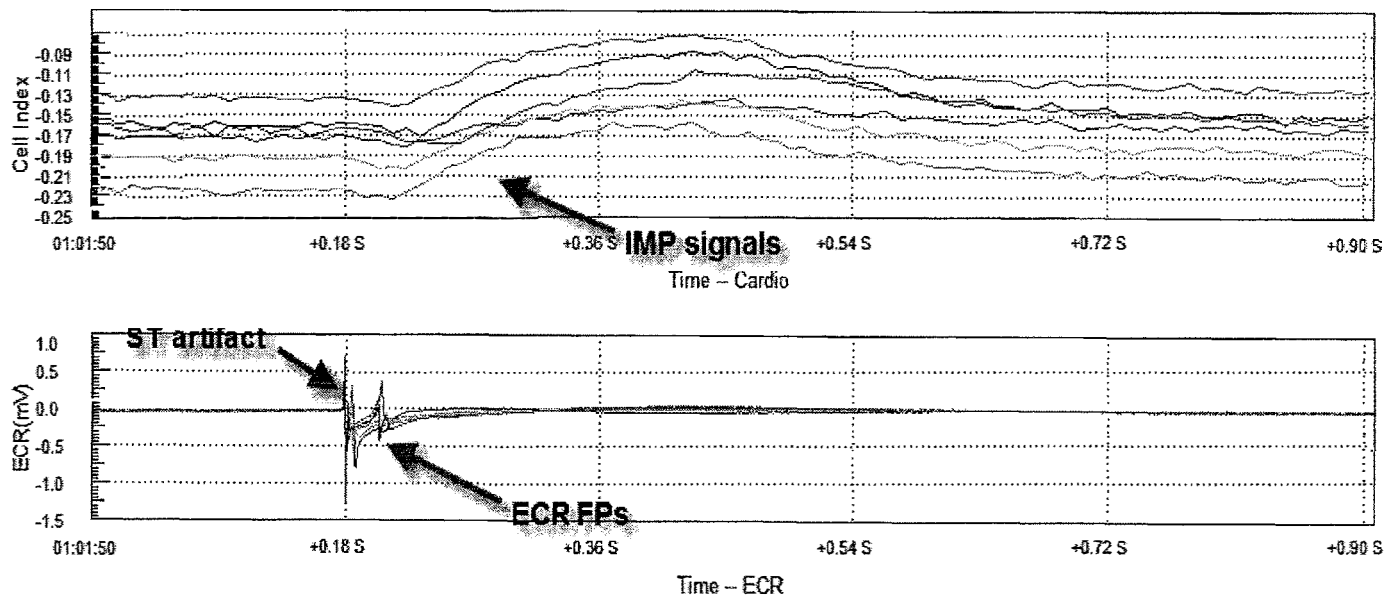


FIG. 13C

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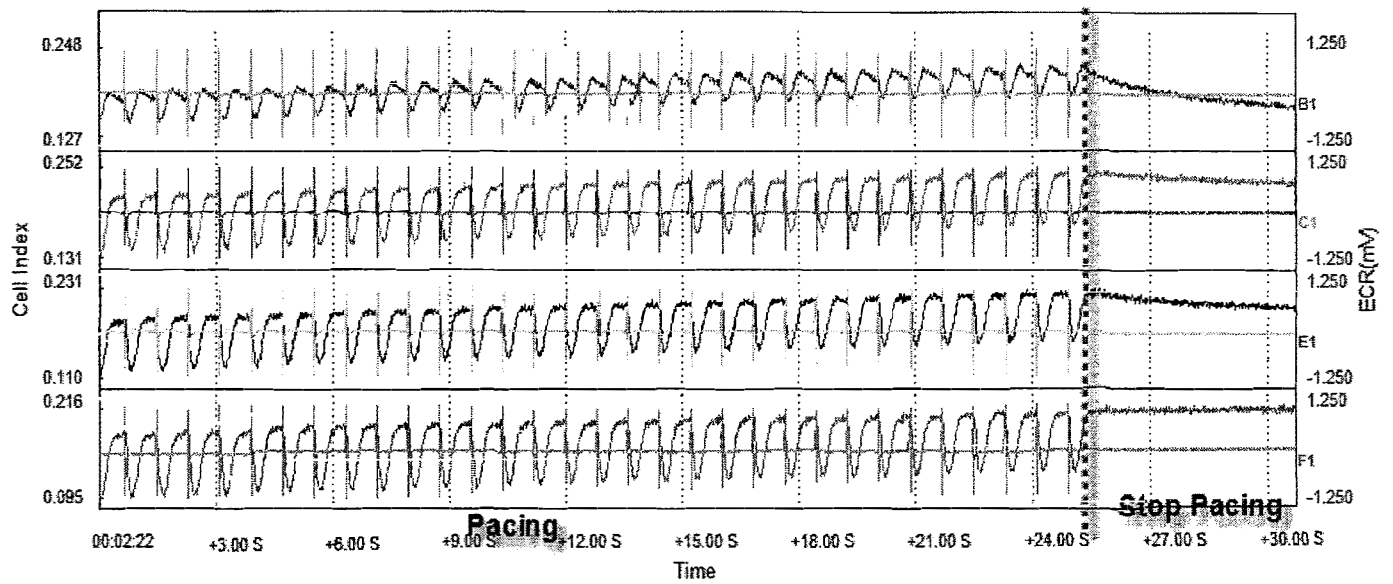


FIG. 14A

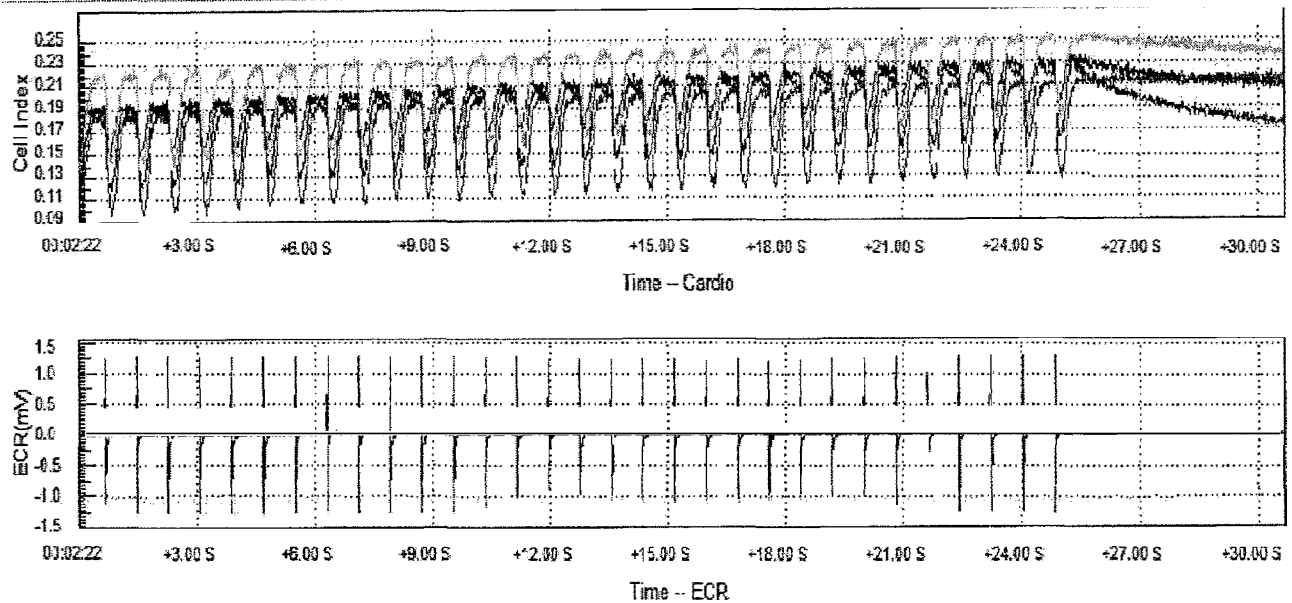


FIG. 14B

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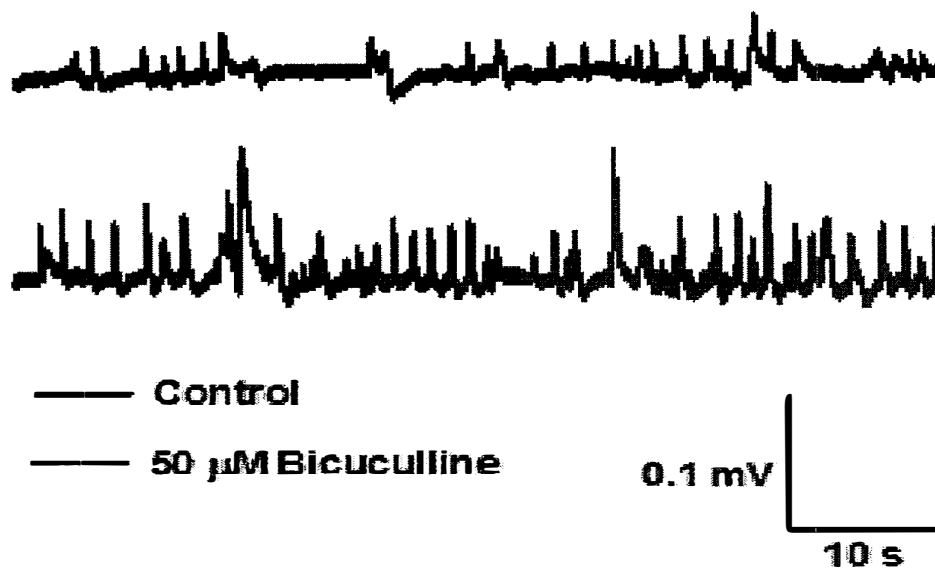


FIG. 15A

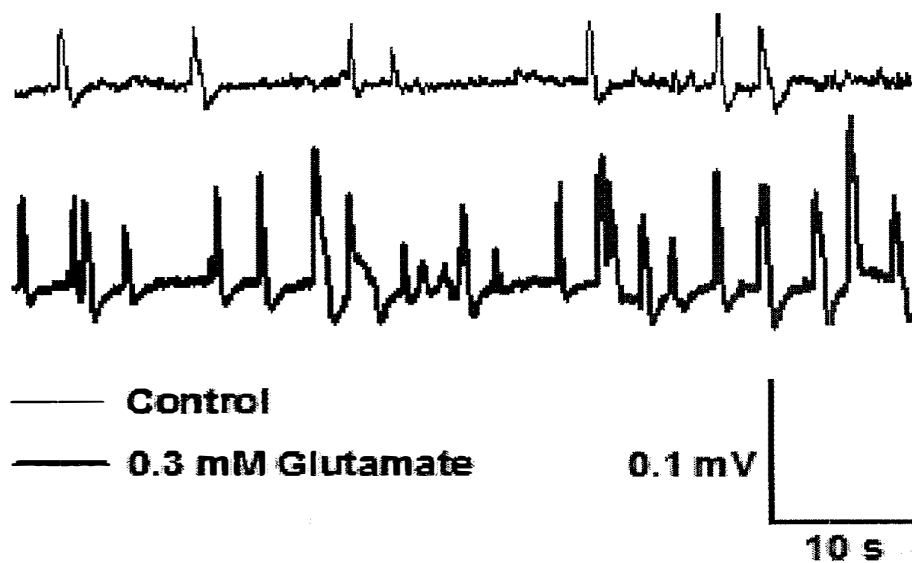


FIG. 15B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/072439

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/02 (2014.01)

USPC - 435/29

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) - C12Q 1/00, 02; C12M 1/00, 34; G01N 27/00, 02, 28, 30, 416, 417, 419, 33/483, 487 (2014.01)

USPC - 435/29, 173.4, 287.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12Q 1/025; G01N 33/4836, 48728, 5061 (2013.01)

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

Orbit, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0039294 A1 (WANG et al) 17 February 2011 (17.02.2011) entire document	1-61
Y	WO 2012/043820 A1 (YASUDA et al) 05 April 2012 (05.04.2012) see machine translation	1-61
A	US 2011/0300569 A1 (LI et al) 08 December 2011 (08.12.2011) entire document	1-61
A	US 2010/0029506 A1 (WANG et al) 04 February 2010 (04.02.2010) entire document	1-61

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search

04 February 2014

Date of mailing of the international search report

19 FEB 2014

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)



(11)

EP 2 291 645 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
09.09.2015 Bulletin 2015/37

(51) Int Cl.:
C12M 1/34 (2006.01) **G01N 33/483** (2006.01)

(21) Application number: **09743426.0**

(86) International application number:
PCT/US2009/042787

(22) Date of filing: **05.05.2009**

(87) International publication number:
WO 2009/137440 (12.11.2009 Gazette 2009/46)

(54) **LABEL-FREE MONITORING OF EXCITATION-CONTRACTION COUPLING AND EXCITABLE CELLS USING IMPEDANCE BASED SYSTEMS WITH MILLISECOND TIME RESOLUTION**

MARKIERUNGSFREIE ÜBERWACHUNG DER ELEKTROMECHANISCHEN KOPPLUNG UND ERREGBAREN ZELLEN ANHAND VON SYSTEMEN AUF IMPEDANZBASIS MIT MILLISEKUNDEN-ZEITAUFLÖSUNG

SURVEILLANCE SANS MARQUEUR D'UN COUPLAGE EXCITATION-CONTRACTION ET CELLULES POUVANT ÊTRE EXCITÉES UTILISANT DES SYSTÈMES FONDÉS SUR L'IMPÉDANCE AVEC UNE RÉOLUTION DANS LE TEMPS DE L'ORDRE DE LA MILLISECONDE

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Description**FIELD OF THE INVENTION**

[0001] The present invention relates generally to impedance based monitoring of cells and more particularly to systems and methods for monitoring excitation-contraction coupling and excitable cells using impedance based systems with millisecond time resolution.

BACKGROUND OF THE INVENTION

[0002] Naichen Yu et al. (Analytical Chemistry 78 (2005), pages 35-43) discloses a system of electrode arrays for monitoring impedance of cells in vitro, wherein two measurements of cell-substrate impedance can be 50 ms apart.

[0003] 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 contraction. Although ECC has been known generally over half a century, it is still an active area of biomedical research.

[0004] 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 patch clamping in an array format, which even though is becoming increasingly available, is not yet widespread. In addition, cardiac toxicity may occur by other mechanisms that may be missed by this type of targeted approach.

[0005] An alternative to 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 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 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 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 and function in vivo are also expressed in ES-derived cardiomyocytes.

[0006] 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. 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 systems typically only monitor a single cardiomyocyte or a small number of cardiomyocytes at a time and with very limited throughput.

SUMMARY OF THE INVENTION

[0007] The present invention addresses the need to provide systems and methods for improved monitoring of excitation-contraction coupling and excitable cells and provides related benefits. Specifically, embodiments of the invention describe

label-free methods for monitoring excitable cells, such as cardiomyocytes, in vitro. The system is capable of continuously monitoring excitation-contraction coupling in a relatively high-throughput manner. The systems and methods can be used for pharmacological safety assessment, screening for novel compounds which modulate cardiomyocyte function in a specific manner, and assessment of genes involved or potentially involved in cardiac function. In addition the system and methods may study the role of different genes and proteins in cardiac function and development through the use of embryonic stem (ES) derived cardiomyocytes.

[0008] In one aspect of the present invention a system for monitoring impedance of excitable cells in vitro is provided including a device for monitoring cell-substrate 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 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. Embodiments of the device for monitoring substrate impedance include a nonconductive substrate having one or more individually addressable electrode arrays fabricated thereon and one or more wells. Preferably, each of the one or more arrays is associated with one of the one or more wells and each electrode array is individually addressable. In further embodiments, a surface of the substrate is suitable for cell attachment, wherein cell attachment results in a detectable change in impedance between electrodes within the array. Attachment or growth along the surface may be enhanced by applying a precoat having one or more compounds that improve attachment. In embodiments where the device includes multiple wells, the impedance analyzer has the capability to allow impedance measurements for each and every well at millisecond time resolution. That is to say, for a selected time period, the impedance data for each and every well of the system is measured at millisecond time resolution. Further, when groups of wells are monitored, the groups are monitored at millisecond time resolution.

[0009] Embodiments of the system include electrode arrays having two electrode structures that have substantially the same surface area. The electrodes themselves may be formed from suitable electrically conductive materials such as metal, gold, platinum, titanium, chromium and the like. The electrode arrays may be organized in a variety of configurations, including interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal, and castellated. The widths of the electrode structures may be between 20 microns and 500 microns or 5 microns and 100 microns.

[0010] The present invention includes impedance monitoring using millisecond time resolution. Millisecond time resolution allows monitoring of at least two consecutive impedance measurements with an excitation cycle of excitable cells, which are introduced into the well. Further, millisecond time resolution enhances measurement such that excitation-contraction coupling of excitable cells may be effectively monitored. In some embodiments the excitation cycle is a beating cycle, such as a beating cycle of a cardiomyocyte.

[0011] In accordance with the invention, at least two consecutive impedance measurements are performed in less than 20 milliseconds. In further embodiments the at least two consecutive impedance measurements are performed in less than 10 milliseconds.

[0012] In some embodiments the device is provided as a multiwell device or a multiwell plate. In some embodiments, the multiwell device or multiwell plate may include 6 wells, 8 wells, 16 wells, 32 wells, 96 wells, 384 wells or any number therebetween. Throughout the present application where the device includes multiple wells each of which is associated with an electrode array, millisecond time resolution refers to that for any selected time period, the impedance measurement for each and every well can be performed with two consecutive measurements separated with millisecond resolution. Further, when groups of wells, such as subsets of all wells of a device, are monitored a group of wells may be monitored using millisecond time resolution.

[0013] Embodiments of the present invention provide impedance-based monitoring with millisecond time resolution in combination with longer term monitoring of cells. By combining millisecond time resolution technology with impedance monitoring, having longer time periods, both short term and long term effects on excitable cells can be evaluated. In some embodiments impedance readouts can be used to monitor both short term beating and long term viability status of cardiomyocytes in the same well or the same population. Such monitoring addresses needs where certain manipulations such as drug treatment may not manifest its effect on cardiomyocyte beating and/or viability until a later time period.

[0014] In some embodiments methods for assessing and quantifying excitable cells in vitro include providing the impedance-based system with millisecond time resolution; adding excitable cells to one or more wells; monitoring impedance of the one or more wells over a first time period, which is characterized as a longer time period, such as seconds, minutes, hours or days; monitoring impedance of the one or more wells over a second time period in millisecond time resolution; determining a characteristic, such as cell attachment, cell growth and cell viability from monitoring over the first time period; and resolving individual cycles of the excitable cells from monitoring over the second time period. In such embodiments, monitoring impedance over the second time period may be performed at regular or irregular time intervals within monitoring impedance over the first time period.

[0015] Among the benefits, embodiments of the invention permit impedance readouts 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.

[0016] In another aspect of the present invention a method for assessing or quantifying excitable cells in vitro is provided. An exemplary method includes providing the impedance based system having millisecond time resolution, adding excitable cells to the one or more wells, monitoring impedance of the one or more wells, and resolving individual cycles of the excitable cells. The method has particular utility with cells that are known or suspected to be capable of or undergo excitation-contraction coupling, such as cardiomyocytes. The method may also be useful for monitoring neurological cells or neurological systems. Impedance measurements are performed in millisecond time resolution, such that two consecutive impedance measurements for each and every well of the system are performed less than 20 milliseconds apart. In further embodiments, at least two consecutive impedance measurements for each and every well of the system are performed less than 10 milliseconds apart.

[0017] In some embodiments methods of determining whether a compound modulates a beating cycle of an excitable cell is provided. An exemplary method includes providing the impedance based system having millisecond time resolution, wherein the system includes at least two wells; adding excitable cells to the at least two wells; monitoring impedance of the at least two wells; adding a compound suspected of modulation of the beating cycle to a first of the at least two wells to provide a test well, wherein a second well lacking compound or having a control compound is provided as a control well; resolving the beating cycle of both test well and control well; and comparing the beating cycles between the test well and control well, wherein a difference in beating cycles indicates the compound modulates the beating cycle of the excitable cell. In some embodiments, beating cycles are compared to identify changes in beating magnitude or amplitude. In other embodiments, beating cycles are compared to identify changes in beating frequency, which may be regular changes or irregular changes. In further embodiments the method includes determining a cell index for each of the test well and control well such that the step of comparing the beating cycles includes comparing the cell indices between test well and control well. Peak magnitude or amplitude and/or frequency may be compared. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance monitoring with intermittent monitoring using millisecond resolution. Thus short term and long term monitoring may be performed.

[0018] In another exemplary methods include providing a device for 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 impedance-based curve or optionally a cell index curve for each of the at least one known biologically active agent 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 may be direct measurement of cardiomyocyte excitation-contraction coupling and if significantly different, concluding that the biologically active agent modulates cardiomyocyte function. 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.

[0019] In other embodiments of the present invention, a method of characterizing rhythmic beating of a cardiomyocyte is provided. An exemplary method includes providing the impedance based system with millisecond time resolution; adding excitable cells to the one or more wells; monitoring impedance of the one or more wells in millisecond time resolution; determining a plurality of beating cycle peaks; and comparing magnitude or amplitude or frequency of the peaks over a time unit. Various methods can be used for determining beating cycle peaks. For 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 first order derivatives of impedance values or cell index values are zero or close to zero in its 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 the data points where the second order derivatives of the impedance values or cell index values is negative and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its 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 is positive and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its absolute value. The step of determining a plurality of beating cycle peaks may include determining a rise in impedance and a decay in impedance and extrapolating a peak from each rise and decay. The method may further include determining a cell index for each of the one or more wells, which may allow improved comparison of beating cycles. Further, beating cycle peaks may be determined by identifying frequency components having largest magnitude in Fourier transformed impedance data. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance

monitoring with intermittent monitoring using millisecond resolution.

[0020] A variety of agents and compounds may be tested for modulation of excitable cells and for modulation of rhythmic beating of cardiomyocytes. Examples include compounds, peptides, proteins, antibodies or antibody fragments, siRNA, shRNA, lipid or any combination of thereof.

[0021] In another aspect of the present invention a method of assessing genetic manipulation of an embryonic stem cell is provided including providing the impedance based system having millisecond time resolution, mutating embryonic stem cells to include a genetic mutation; adding mutated embryonic stem cells to a first well of at least two wells to form a test well and control embryonic stem cells to a second of at least two wells to form a control well; monitoring impedance of the at least two wells in millisecond time resolution; resolving the beating cycles of both test well and control well, if any; and comparing the beating cycles between the test well and control well, wherein a difference in beating cycles indicates the genetic mutation modulates embryonic stem cell differentiation. In some embodiments the embryonic stem cell differentiation is differentiation into a cardiomyocyte. In some embodiments the genetic mutation is selected from the group consisting of a genetic knock out, a knock down and an inserted transgene. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance monitoring with intermittent monitoring using millisecond resolution. Thus short term and long term monitoring may be performed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Those skilled in the art will recognize that the drawings described below are for illustrative purposes only. The drawings are not intended to limit the scope of the invention but to provide exemplary embodiments.

FIG. 1 depicts schematic drawings of one design of a cell-substrate impedance measurement device of the present invention. **FIG. 1A** shows a nonconductive substrate (**101**) shown with 16 electrode arrays fabricated on the substrate. Each electrode array (**102**) comprises two electrode structures (shown in detail in **FIG. 1B**). Each electrode 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 the four electrode arrays in each of four quarters on the substrate (**101**) have one of their electrical connection traces (**103**) connected to a common connection pad (**104**). Thus, for the entire 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**). In **FIG. 1B** a single exemplary electrode array 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 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. 2A is a schematic representation of a device **100** with two electrode structures of same or similar areas deposited on a substrate. First electrode structure has electrode elements **110a**, **110b**, **110c** and second electrode structure has electrode elements **120a**, **120b**, **120c** and **120d**. Electrode elements within an electrode structure are connected to each other by arc-shaped connection electrode bus **125**. Like the electrode elements, such connection-buses (**125**) are also made of electrically-conductive material (e.g. gold film, platinum film, gold film over a chromium or titanium film). These electrically-conductive connection-paths or connection buses (**125**) may have an insulating coating. Electrode elements **110a-110c** and **120a-120d** comprise electrode lines with connected circles added on the line. The overall area of electrode elements and gaps between electrode elements may correspond to, or may be slightly larger than, or may be slightly smaller than, the bottom of a well (e.g., a cylinder shaped well, a conical shaped well, or a cubic shaped well), for example, a 24 well-plate, a 96-well plate, or 384 well plate that are commonly used. The whole surfaces of the wells may be covered with electrodes to ensure that the cell attachment at nearly any locations of the bottom surface of the well can contribute to the impedance change. This arrangement has an advantage that non-uniform landing and attachment of cells on the bottom surface of the different wells would result in only a small variation in the impedance measured between electrode structures **110** and **120**. **150** are connection pads that can be connected to an external impedance measurement circuit. **130** is the electrical connection traces that connects the connection pad to the electrode structures **110** and **120**. Such connection traces can extend in any direction in the plane of the electrodes. **FIG. 2B** is a schematic representation of a device **200** with two electrode

structures of similar areas deposited on a substrate. Electrode structures **210** and **220** comprise multiple interconnected electrode elements. Electrode elements (**210a-210c**, **220a-220d**) are rectangular lines and together form an interdigitated electrode structure unit. Similar to **FIG. 2A**, the electrode elements (**210a-210c**, **220a-220d**) within each electrode structure are connected through arc-shaped, electrically conductive paths or electrode buses (**225**). Connection pads **250** are connected to electrode structures through the electrical connection traces **230**. **FIG. 2C** is a schematic representation of a device **300** with two electrode structures of similar areas deposited on a substrate. Electrode structures **310** and **320** comprise multiple interconnected electrode elements (**310a-310f**, **320a-320f**). Electrode elements (**310a-310c**, **320a-320d**) are rectangular lines and together form an interdigitated electrode structure unit. Different from **FIG. 2A** and **FIG. 2B**, the electrode structures having electrode elements **310a-310c** and **320a-320c** are connected to connection pads **350**.

FIG. 3 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. In this example, the impedance measurement was taken with a time resolution of 1 hour between any two consecutive data points for each well.

FIG. 4A provides a plot of normalized cell index values for cells in different wells treated with different concentrations of sodium dichromate dehydrate (SDD). **FIG. 4B** provides the corresponding sigmoidal curve for deriving the IC-50 of the compound.

FIG. 5A provides a graph of normalized impedance data over time showing shifts in morphology in response to isoproteranol, a β_2 adrenergic receptor agonist.

FIG. 5B shows a plot of the normalized cell indices against the log of the corresponding isoproteranol concentrations to calculate the EC-50.

FIG. 6 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 long term measurement, cell electrode impedance and corresponding cell indices were measured at about 15 minute intervals. Thus for each well, the impedance measurement was performed with a time resolution of 15 minutes.

FIGS. 7A-D provides a series of graphs showing cardiomyocyte beating throughout attachment and growth phases.

FIG. 8A and **FIG. 8B** are a pair of cell index curves and the corresponding beat rate derived from Fourier transformation of impedance values

FIGS. 9A-D show cardiomyocyte beating data from mouse cardiomyocytes treated with sotalolol. In **FIGS. 9C** and **9D**, the time resolution between two adjacent points is 40 milliseconds. In other words, a second in **FIGS. 7C** and **7D** is equivalent to 40 milliseconds.

FIG 10A depicts graphs of agents which slow down heart rate and cardiomyocyte beating. **FIG. 10B** depicts graphs of agents which increase heart rate and cardiomyocyte beating.

FIGS. 11A-S depict impedance data of cardiomyocyte patterns associated with testing of a variety of known compounds, which are further described in **Table II**.

FIG. 12 depicts impedance based cardiotoxic patterns from known compounds.

FIG. 13 shows an example of dose dependent effects of Astemizole on cardiomyocytes beating at different concentrations.

FIGS. 14A - H are graphical depictions of real time impedance-based millisecond time resolution of cardiomyocyte beating in response to Haloperidol treatment. The Y-axis is the cell index value.

FIGS. 15A-H depict graphical analysis of the impedance beating frequency from impedance plots (cell index plots) shown in **FIGS. 14A - H**.

FIGS. 16A-H depict additional graphical analysis of the magnitude or amplitude of the beating cycle peaks from impedance plots (cell index plots) shown in **FIGS. 14A-H**.

FIGS. 17A-I are graphical depictions of real time impedance-based millisecond time resolution of cardiomyocyte beating in response to Erythromycin treatment. The Y-axis is the cell index value.

FIGS. 18A-I depict graphical analysis of the impedance beating frequency from impedance plots (cell index plots) shown in **FIGS. 17A - I**.

FIGS. 19A-I depict additional graphical analysis of beating magnitude or amplitude of the beating cycle peaks shown from impedance plots (cell index plots) shown in **FIGS. 17A - I**.

DETAILED DESCRIPTION OF THE INVENTION

[0023] Detailed descriptions of preferred embodiments of the provided herein. It is to be understood; however, that the present invention may be embodied in various forms. Therefore specific reference to various forms are provided as a basis for the claims and for teaching one skilled in the present art to employ the present invention in appropriate system, structure or manner.

A. Definitions

[0024] Unless defined 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 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.

[0025] As used herein, "a" or "an" means "at least one" or "one or more."

[0026] As used herein, "membrane" is a sheet of material.

[0027] As used herein, "biocompatible polymer" means a polymeric material that does not have deleterious effects on cells, including the viability, attachment, spreading, motility, growth, or cell division.

[0028] As used herein, "suitable for attachment" refers to structural features that permit attachment of cells thereto. A surface is "suitable for cell attachment" when a significant percentage of the cells attach or adhere to the surface within twelve hours. Preferably, at least 50% of the cells are adhered to the surface within twelve hours. More preferably, at least 70% of the cells are adhered to the surface within twelve hours of plating (i.e., adding cells to the well or fluid container). 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 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. To have desired surface properties for cell attachment, the surface may need to be chemically-treated (e.g. treatment with an acid and/or with a base), and/or physically treated (e.g. treatment with plasma), and/or biochemically treated (e.g. coated with one or more molecules or biomolecules that promotes cell attachment).

[0029] As used herein, "impedance measurement at millisecond time resolution" refers to the ability to perform a series of at least two consecutive impedance measurements within milliseconds of one another for each and every well of the system where each well is associated with an electrode array for impedance measurement. In each instance the time between two consecutive impedance measurements are less than 300 milliseconds from one another. Preferably, consecutive impedance measurements are less than 200 milliseconds from one another. More preferably impedance measurements are less than 100 milliseconds from one another. For example, the time between two consecutive impedance measurements are 40 milliseconds from one another for each and every well of the system. To be clear, that is to say, in a time period of 40 milliseconds, the system has the ability to measure two impedance data points for each and every well of the system.

[0030] As used herein, "excitable cells" refers to a cell population that is capable of propagation and spreading action potentials to surrounding cells. These cells are able to produce and respond to electrical signals. Some excitable cells are capable of excitation-contraction coupling. Examples of "excitable cells" include cardiomyocytes, cells that beat, some skeletal muscle cells, some smooth muscle cells and some neural cells. Identification of excitable cells may be found in cell biology literature.

[0031] As used herein, "excitation-contraction coupling" refers to the physiological process of converting an electrical stimulus to mechanical response. This process is fundamental to muscle physiology, whereby the electrical stimulus is usually an action potential and the mechanical response is contraction. EC coupling can be dysregulated in many disease conditions.

[0032] As used herein, "beating cycle" refers the time between which a cardiomyocyte sequentially beats. The "beating cycle" may vary depending on organism and developmental stage of the cardiomyocyte. A beating cycle may be as

short as between 100 and 200 milliseconds, translating to a beating rate of 600 to 300 beats per minute. A beating cycle may be as long as between 1000 milliseconds and 10,000 milliseconds, translating to a beating rate of 60 to 6 beats per minute.

[0033] As used herein, "biomolecular coating" or "coated with a biomolecule" refers to is a coating on a surface that comprises a molecule that is a naturally occurring biomolecule or biochemical, or a biochemical derived from or based on one or more naturally occurring biomolecules or biochemicals. For example, a biomolecular coating can comprise an extracellular matrix component (e.g., fibronectin, collagens), or a derivative thereof, or can comprise a biochemical such as polylysine or polyornithine, 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.

[0034] As used herein, "extracellular matrix component" refers to 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.

[0035] As used herein, "electrode" refers to a structure having a high electrical conductivity, that is, an electrical conductivity much higher than the electrical conductivity of the surrounding materials.

[0036] As used herein "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.

[0037] 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.

[0038] As used herein "electrode structure unit" refers to 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.

[0039] As used herein, "electrode traces" refer to 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 impedance analyzer. The end or boundary of a device may correspond to the connection pads on the device or apparatus.

[0040] As used herein, "connection pad" refers to 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). The electrical connection between a connection pad and an impedance measurement 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.

[0041] As used herein, "interdigitated" refers to having projections coming from 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).

[0042] As used herein, "high probability of contacting an electrode element" refers to 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 on 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%.

[0043] As used herein, "at least two electrodes fabricated on said substrate" refers to 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.

[0044] As used herein, "at least two electrodes fabricated to a same side of said substrate" refers to at least two electrodes are fabricated on the same side of the substrate.

[0045] As used herein, "at least two electrodes fabricated to a same plane of said substrate" refers to, if the nonconducting substrate has multiple layers, the at least two electrodes are fabricated to the same layer of the substrate.

[0046] As used herein, "electrodes have substantially same surface area" refers to 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 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 referred to. In other

words, where electrodes 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 structure is less than 5, 4, 3, 2, 1.5, 1.2 or 1.1. More preferably, the at least two electrodes of an electrode structure have nearly identical or identical surface area.

[0047] As used herein, "said device has a surface suitable for cell attachment or growth" refers to 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 necessary substances, e.g., nutrients or growth factors, can be supplied in a medium. Preferably, when a suspension of viable, unimpaired, excitable cells is added to the "surface suitable for cell attachment" at least 50% of the cells adhere to the surface within twelve hours. More preferably, a surface that is 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 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.

[0048] As used herein, "detectable change in impedance between or among the electrodes" refers to the impedance between or among the electrodes would have a significant change that can be detected by an impedance analyzer or impedance measurement circuit when molecule binding reaction or cell attachment or cell adhesion or cell presence occurs on the electrode surfaces. The impedance change refers to the difference in impedance values when cell attachment or cell adhesion or cell presence or molecule binding reaction occurs on the electrode surface of the apparatus and when no molecular reaction occurs on the electrode surface or no cell is present on the electrode surface. The impedance change may occur upon the presence or decay of a beat from a cardiomyocyte. Alternatively, 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 surface, or when the number, type, activity, 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 the 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 the 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 the 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 the 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.

[0049] As used herein, "multiple pairs of electrodes or electrode structures spatially arranged according to wells of a multi-well microplate" refers to 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.

[0050] As used herein, "arranged in a row-column configuration" refers to 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.

[0051] As used herein, "each well contains substantially same number... of cells" refers to 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 well contains an identical number of cells.

[0052] 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 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. In some instances the "same type of cells" may initially refer to embryonic stem cells; however, the stage of differentiation into cardiomyocytes may be differ.

[0053] 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.

[0054] 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 be cardiotoxic if it impairs cardiomyocyte beating, whether magnitude or amplitude or frequency, or if it causes cell death. Then the response of cells can be measured by percentage of non-viable (or viable) cardiac cells after the cells are treated by the test compound.

[0055] As used herein, "microelectrode strip or electrode strip" refers to 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 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, circle-on-line electrodes, diamond-on-line electrodes, castellated 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 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.

[0056] 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, modulating the beating cycle of a cardiomyocyte cell population, whether by beating amplitude or frequency. 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).

[0057] 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 cardiotoxicity or embryonic stem cell development, etc.

[0058] An "impedance value" is the impedance measured for electrodes in a well with or without cell 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.

[0059] 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.

[0060] 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.

[0061] 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.

[0062] 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

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

[0063] As used herein, "primary cell" or "primary cells" refers to any non-immortalized cell that has been derived from various tissues and organs of a patient or an animal.

B. Introduction to Impedance-Based Systems and Methods having Millisecond Time Resolution

[0064] The impedance-based system and methods of the present invention fill a major technological gap in monitoring of excitation-contraction coupling and excitable cells, such as cardiomyocytes, in vitro. This is accomplished, in part, from the development of an impedance-based system and monitoring method that employs millisecond time resolution. Millisecond timer resolution permits effective monitoring of excitable cells for short durations, such as less than about 100 milliseconds. Further by coupling millisecond time resolution with longer duration time periods, both short term and long term effects of drugs, such as cell viability, changes in morphology and adhesion can also be assessed. These combination studies permit further data collection with respect to compounds such as β -2 adrenergic receptor agonists, well known and characterized modulators of heart function in vivo and in vitro, which can induce long term hypertrophic responses in cardiomyocytes, which is associated with elongated morphology of the cells. When combining long term monitoring and short term monitoring it may be desirable to provide long term monitoring coupled to intermittent short term monitoring periods.

[0065] The devices, systems and methods provided permit impedance-based monitoring of excitable cells using millisecond time resolution. It has been discovered that by developing enhanced impedance based systems, detailed analysis of excitable cells, the excitation cycle of cells and potential modulators of excitable cells can be performed. Further, by studying the excitation cycle with increased resolution embodiments of the invention permit the effective evaluation of factors that may alter normal or abnormal cycling. Without millisecond time resolution, it would not be possible to perform detailed analysis of the beating cycle of the excitable cells. For example, for cardiomyocytes, if the impedance is measured at seconds time-resolution such as a ten-second or even one-second resolution, it would not be possible to observe beating of the cardiomyocytes or resolve a beating cycle of the cardiomyocytes with an impedance measurement. The cardiomyocytes-beating-resulted impedance change would be shown as "noise" on the impedance curves measured at seconds or minutes time-resolution. Indeed, it was a surprise that the beating of the cardiomyocytes on the electrode surfaces would result in periodic change in impedance and even more surprise that the impedance measurement, when performed at appropriate time resolution of milli-seconds, could resolve such beating cycles. After we discovered that cardiomyocyte beating, when cultured on electrode surfaces, can be monitored via impedance measurement, we interpreted that such dynamic monitoring of cardiomyocyte beating is based on quantification in real time of the rhythmic changes in cardiomyocyte morphology and/or attachment as a result of the excitation contraction coupling of the electrically excitable cardiomyocytes growing on microelectrodes' surface in E-PLATES (ACEA Biosciences, San Diego, CA). The quantification of the rhythmic changes in cardiomyocyte morphology and/or attachment is achieved via the fast (millisecond resolution) 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.

[0066] When combining long term impedance monitoring and short term monitoring it may be desirable to provide long term monitoring coupled to intermittent short term monitoring. In other words, while long term impedance monitoring is able to identify long term effects on excitable cells, short term impedance monitoring, such as at millisecond time resolution may be employed at particular time points to assess short term effects. In some instances, impedance mon-

itoring with millisecond time resolution is performed at regular or irregular time intervals. In these instances, time intervals may be preprogrammed in the system or may be initiated by the user. In some instances intermittent measuring at millisecond time resolution is initiated upon the occurrence of an event, such as an event detected or determined while long term monitoring of impedance. Such events may be changes in measured impedance data, changes in optical property and the like. Monitoring at millisecond time resolution may continue until a programmed event occurs, such as expiration of a time period. After or during which, long term impedance monitoring may continue.

[0067] In some embodiments, long term and short term monitoring of excitation-contraction coupling or excitable cells is provided in combination. Exemplary methods include providing the impedance-based system with millisecond time resolution; adding excitable cells or cells suspected of excitation-contraction coupling to one or more wells; monitoring impedance of the one or more wells over a first time period, which is characterized as a longer time period, such as seconds, minutes, hours or days; monitoring impedance of the one or more wells over a second time period in millisecond time resolution; determining a characteristic, such as cell attachment, cell growth and cell viability from monitoring over the first time period; and resolving individual cycles of the excitable cells from monitoring over the second time period. In such embodiments, monitoring impedance over the second time period may be performed at regular or irregular time intervals within monitoring impedance over the first time period. Further, test agents may be added to assess their short term effects and long term effects on the cells.

[0068] Impedance monitoring using, at least in part, millisecond time resolution will be useful in the testing of compounds such as drugs for the treatment or prevention of various medical conditions or their safety. In some embodiments the systems and methods are used to test or evaluate potential compounds, drugs or treatments for cardiotoxicity data. In some embodiments potential compounds, drugs or treatments are tested for cardioprotective data. In some embodiments potential compounds, drugs or treatments are tested for potential applicability for cardiovascular treatments, including hypertension or congestive heart failure. In some embodiments potential compounds, drugs or treatments are tested for modulation of embryonic stem cell development, such as development into cardiomyocytes. In other embodiments the systems and methods are used to test or assess potential drugs or treatments for neurological data, such as beneficial or deleterious neurological effects. As will be understood by those skilled in the present art, the impedance based system and methods including millisecond time resolution permits evaluation across multiple cell populations or systems that including excitation-contraction coupling.

[0069] One skilled in the art will understand embodiments of the present invention may be used for studying a variety of excitable cells or effects thereon. As nonlimiting examples cells may be cardiac muscle cells, neurological cells, differentiating stem cells, embryonic stem cells, adult stem cells and the like. Smooth muscle cells and skeletal muscle cells that undergo excitation-contraction coupling may also be studied.

[0070] In some embodiments of the present invention the excitation cycle or beating of cardiomyocytes is studied. Cardiomyocytes are specialized muscle cells, which make up the majority of the volume of the myocardium. The myocardium also includes epithelial cells and fibroblasts. Cardiomyocytes are arranged largely in a circumferential and spiral orientation around the left ventricle, the chamber that pumps blood to the systemic circulation. Atrial myocytes, also cardiomyocytes, are smaller in diameter and less structured than their ventricular counterparts. Cardiomyocytes have five major components: cell membrane (sarcolemma) and T-tubules, for impulse conduction; sarcoplasmic reticulum, a calcium reservoir needed for contraction; contractile elements; mitochondria; and nucleus. Cellularly, cardiomyocytes may be identified by the presence of cardiac troponin T (TnT) and atrial natriuretic peptide (ANP).

[0071] The functional intracellular contractile unit of cardiac muscle (like skeletal muscle) is the sarcomere, an orderly arrangement of thick filaments composed principally of myosin, and thin filaments containing actin. Contraction of cardiac muscle occurs by the cumulative effort of sliding of the actin filaments between the myosin filaments toward the center of each sarcomere.

[0072] Cardiomyocytes may be obtained or isolated from cardiac tissue directly, may be cultured from cardiomyocyte cells or may be differentiated from stem cells, such as embryonic stem cells. In some embodiments embryonic or adult stem cells are used. Methods for obtaining or isolating cardiomyocytes from cardiac tissue may be found in the literature.

[0073] Since embryonic stem (ES) cells are self renewing cells in culture they can serve as an excellent source for continuous production of cardiomyocytes. These derived 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. 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 concern of adverse affects on overall embryonic development in transgenic animals. Further, the ability to 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 express the GFP transgenes and can be easily identified and isolated by cell sorting techniques and therefore an enriched cardiomyocyte population can be obtained.

C. Impedance-Based Systems Having Millisecond Time Resolution

[0074] In preferred embodiments the system for monitoring impedance of excitable cells in vitro includes a device for monitoring cell-substrate 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 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 impedance based system with millisecond time resolution, excitation-contraction coupling cells can be efficiently monitored and studied. Accordingly, the systems provided herein are able to identify and evaluate changes in excitation-contraction events, which may be used for high throughput analysis of potential therapeutics. 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.

[0075] Embodiments of the device for monitoring substrate impedance include a nonconductive substrate having one or more individually addressable electrode arrays 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 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 and urea resin. Preferably, the substrate is biocompatible with excitable cells; 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 precoating the substrate with a protein or compound that facilitates 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.

[0076] Each electrode array includes 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. 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 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.

[0077] 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 by using switches (such as electronic switches).

[0078] 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 does 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 does 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 does 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 does not differ by more than 2%.

[0079] 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.

[0080] 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.

[0081] 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.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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.

[0086] Descriptions of electrode arrays used for impedance measurement that apply to the devices of the present invention are 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.

[0087] 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 the opposite electrode structure. Electrode arrays may be provided in configurations, such as interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal and castellated.

[0088] Preferably, the electrode elements (or electrode structures) of an array of the present device of the present invention are of approximately equal widths. Preferably 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.

[0089] Preferably, the electrode elements (or electrode structures) of an array of the present device of the present invention are approximately evenly spaced. Preferably, 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.

[0090] A device of the present invention can include one or more fluid-impermeable receptacles which serve as fluid containers or wells. Such receptacles may be reversibly or 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 Application 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.

[0091] 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.

[0092] 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.

[0093] 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.

[0094] In the system for monitoring impedance of excitable 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 analyzer is capable of measuring impedance between 1 ohm and 10^3 ohm in frequency range of 1.00 Hz to 300 kHz.

[0095] 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 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 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 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 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.

[0096] Millisecond time resolution of impedance-based devices provides a significant technical step over traditional impedance measuring devices, which are typically limited to second or minute resolution. That is, substantial reconfig-

uration of a traditional impedance-based system is required to obtain millisecond time resolution. Advances in multiple steps are required to achieve millisecond time resolution, including the impedance measurement circuitry together with electronic switching circuitry. Technological advances are further required when performing millisecond time resolution over 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, for a given time period, measuring the impedances of all 96 wells with millisecond time resolution between two adjacent or consecutive impedance measurement points for each and every well. Thus, comparing two impedance analyzers, one for impedance measurement of 96 wells with millisecond resolution and another for impedance measurement of a single well with millisecond time resolution, the one for 96 well measurement is effectively 96-times faster than one for a single well measurement since it is required to have a capability for performing impedance measurement for all 96 wells with millisecond time resolution. Similarly, if a 96 well device is provided, a group of wells, such as a group of 2, 4, 6, 12, 24 or 48 wells, may be measured in millisecond time resolution. 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. 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.

[0097] 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 millisecond time resolution. For instance, improvement likely requires the use of fast processing electronic chips for analogue-to-digital conversion, for parallel digital signal processing and data calculation with field-programmable gate array (FPGA) and for fast communication between the impedance measurement circuitry and software. Another example 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 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 millisecond resolution. And, a very important is to replace previous working mode of "measurement of one-well's impedance at a time" with a mode of "measurement of multiple-wells' impedances at a time". In "one-well 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 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' impedances. The measurement 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 the same time or simultaneously with more than one well's data being sent at a time. In this "measurement of multiple-wells' impedances 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.

[0098] With millisecond time resolution for impedance measurement, it becomes 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.

[0099] 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 switching between occur 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.

[0100] 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.

[0101] 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.

[0102] 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.

[0103] 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.

[0104] 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.

[0105] Preferably, the software can also analyze impedance data. In preferred embodiments, the software can calculate a cell index (CI) for one or more time points for one or more wells of the multiwell device. In some preferred embodiments, the software can calculate a cell change index (CCI) from impedance measurements of one or more wells of the multiwell device. The software can preferably generate plots of impedance data and impedance values, such as but not limited to CI or CCI, with respect to time. The software may perform other analysis as well, such as calculate cell number from CI, generate dose-response curves based on impedance data, calculate IC values based on impedance values, and calculate kinetic parameters of the excitation cycle cell based on impedance values and impedance value curves. In some embodiments the beating cycle of a cardiomyocyte population is determined, which may include initiation and decay of individual beats. Peaks may be derived from the detection of vectors associated with initiation of beating or beating decay. Peaks may be derived with other methods. In further embodiments the change in beating cycle of a cardiomyocyte population is determined in response to a stimulus such as a pharmacological agent. The software of the impedance monitoring system can also store and display analyses of the data, such as calculated impedance values and kinetic parameters derived therefrom. Data can be displayed on a screen, as printed data, or both. Data may be stored on a hard drive for exportation into compatible programs for further analysis or data storage.

[0106] Further 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 no. 11/235,938, 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 each is briefly summarized.

[0107] The cell index obtained for a given well reflects: 1) how many cells are attached to the electrode surfaces in this well, 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 cell index = 0. A higher value of "cell number index" indicates that, for 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 same type of the cells and 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.

[0108] 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.

[0109] 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.

[0110] 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.

[0111] Thus CCI is the normalized rate of change in cell index. CCI values can be used to quantify the cell status change. For cells in an exponential growth under regular cell culture condition, the cell index determined by a cell-substrate impedance monitoring system described herein is expected to be a proportionate measure of the cell number in the well since the cell morphology and average extent of cell adhesion to the electrode surfaces among the whole cell population do not exhibit significant changes over time.

[0112] After measuring and recording time dependent impedance data for excitable cells cultured on electrodes, various methods can be used for determining beating cycle peaks. For 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 first order derivatives of impedance values or cell index values are zero or close to zero in its 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 the data points where the second order derivatives of the impedance values or cell index values is negative and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its 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 is positive and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its absolute value. In another example, the 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 are the magnitude or amplitude of the beating cycle peaks.

[0113] After determining the beating cycle peaks, various methods can be used to calculate the beating rate. The beating rate can be determined by dividing a unit time (e.g., 1 minute) by the time period between two adjacent peaks. For example, if two adjacent peaks are separated by 500 milliseconds, then the beating rate for this 500 milliseconds can be determined as 120 beats per minute. Another method is simply to count how many peaks in a unit time by software. For example, if there are 2 peaks in one second interval, then the beating rate should be 2 beats per second, or 120 beats per minute.

D. Assessing or Quantifying Excitable Cells, Excitation Contraction-Coupling and Cardiomyocytes In Vitro

[0114] In another aspect of the present invention a method for assessing or quantifying excitable cells in vitro is provided. The methods including detecting or monitoring excitable cells using impedance monitoring with millisecond time resolution. In preferred embodiments, the method includes providing the impedance based system having millisecond time resolution system, adding excitable cells to one or more wells of the device, monitoring impedance of the one or more wells with millisecond time resolution, and resolving individual cycles of the excitable cells. In some embodiments monitoring impedance further includes determining cell indices from impedance values, calculating average rate of excitation events per unit time (such as beats per unit time when using cardiomyocytes), determining amplitude intensity or average amplitude intensity in a unit time, and/or determining the length of time between excitation cycles (or beating cycles). These additional determinations may be compared to further assess excitable cells.

[0115] The methods have particular utility with cells that are known or suspected to be capable of or undergo excitation-contraction coupling, such as cardiomyocytes. Thus the methods may monitor, detect or determine excitation contraction-coupling of excitable cells. Further, even minor variations in excitation-contraction coupling may be detected.

[0116] Preferably impedance measurements are performed with millisecond time resolution. In some embodiments, two consecutive impedance measurements are performed at less than 300 milliseconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 100 seconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 40 milliseconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 10 milliseconds apart. In some embodiments at least two impedance measurements are between 1 millisecond and 50 milliseconds apart.

[0117] Cardiomyocytes are a subset of cells that are involved in excitation-contraction coupling. Embodiments of the present invention include applications for studying cardiomyocyte activity, function and its modulation. By monitoring the continuous rhythmic beating of cardiomyocytes, methods of the present invention allow the study of cardiomyocytes themselves as well as screening for pharmacological agents which may alter cardiomyocyte rhythmic beating. Thus the methods permit screening of compounds or conditions that affect cardiomyocyte function or induce cardiotoxicity. Specifically, methods may assess or quantify cardiomyocyte beating in vitro using impedance-based technology having millisecond time resolution. In preferred embodiments, the methods are based on quantification in real time 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 E-PLATES (ACEA Biosciences, Inc. San Diego, CA). Thus quantification of the rhythmic changes in cardiomyocyte morphology is achieved via the measurement of electrode impedance, which may be performed with millisecond time resolution. In summary, some methods provide a cellular cardio-gram, which can provide incisive information about the status of cardiomyocytes especially upon treatment with pharmacological agents.

[0118] Cardiomyocytes used in the present invention may be any known or experimental cardiomyocyte population. Human cardiomyocytes will be particularly desirable when studying the effect of pharmacological agents for human use; however, cardiomyocytes may also be murine or mouse, rat, rabbit, dog, and the like. Further, the cardiomyocytes may be primary cardiomyocytes isolated from cardiac tissue or may be cultured cardiomyocytes, which are cultured over time. In some embodiments, ES cells destined to differentiate into cardiomyocytes are used. Isolated primary cardiomyocytes as well as ES-derived cardiomyocytes retain the ability to beat in culture. Thus, these cells provide a desirable model system to study cardiomyocyte function in vitro.

[0119] Cardiomyocytes have an innate ability to undergo mechanotransduction. In other words, the spontaneous force generated from 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 system can sensitively and precisely detect transient changes in morphology and adhesive capacity of the cells, it can be used to monitor cardiac function in vitro. Further, the development of millisecond time resolution permits impedance monitoring of the cardiomyocyte beating cycle as well as fluctuations, even minor alterations, in response to external stimuli.

[0120] Exemplary steps involved in using an impedance-measurement system for measurement of cardiomyocyte function include: providing a system for monitoring impedance of excitable cells, in vitro, which is capable of millisecond time resolution, optionally coating wells of the device with either fibronectin or other matrix proteins; seeding either embryonic stem cells (ES cells) of mammalian origin or primary cardiomyocytes at sufficient seeding densities into the wells of the device; allowing the cells to attach and spread; after a period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

[0121] The millisecond kinetic readout requires the impedance measurement system be capable of obtaining impedance measurement data at millisecond time resolution. Preferably, the time difference between two consecutive impedance measurements for a well is within the range of milliseconds (e.g., less than 300 milliseconds, less than 100

milliseconds, less than 50 milliseconds, less than 20 milliseconds, less than 10 milliseconds, or less than or about 1 millisecond). Due to the fast nature of beating cycles of cells, millisecond kinetic readout is required for proper resolution. Thus, the time resolution for the impedance measurement should allow the system to perform, measurement using at least two time points for each beat cycle, or at more than two points for each beating cycle. In some embodiments, three, four, five or even ten, or fifteen or twenty or more time points within each beating cycle are obtained.

[0122] Embodiments of the present invention include methods of assessing or quantifying cardiomyocytes include providing the system for monitoring impedance of excitable cells in vitro, which includes millisecond time resolution, adding cardiomyocyte to one or more wells, monitoring impedance of the one or more wells, such as using millisecond time resolution, and resolving excitation cycle or beating cycle of the cardiomyocytes. In further embodiments, the method also includes determining cell indices from impedance values, 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. In preferred embodiments, millisecond time resolution permits measurement of at least two consecutive impedance measurements within a beating cycle. In some embodiments at least three consecutive impedance measurements are performed with millisecond time resolution.

[0123] 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 system can sensitively and precisely detect transient changes in morphology and adhesive capacity of the cells, the it can be used to monitor cardiomyocyte beating in vitro.

[0124] Another exemplary method for impedance based measurement of cardiomyocyte beating includes: providing 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; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding either embryonic stem cells (ES cells) of mammalian origin or primary cardiomyocytes at specific seeding densities to the wells of the device; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

[0125] In another exemplary embodiment for measurement of cardiomyocyte function the method includes: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding either ES cells of mammalian origin or primary cardiomyocytes at specific seeding densities; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using an impedance-based system by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

[0126] Dynamic monitoring of cardiomyocyte beating is based on quantification in real time of the rhythmic changes in cardiomyocyte morphology as a result of the excitation contraction coupling of the electrically excitable cardiomyocytes growing on microelectrodes' surface of E-PLATES. The quantification of the rhythmic changes in cardiomyocyte morphology is achieved via the fast, millisecond resolution 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. An exemplary method includes providing a device for measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least one well optionally coated with fibronectin 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 at time intervals over a period of time and optionally determining cell indices from impedance values; 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.

[0127] 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 β -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 impedance-based system can be used to distinguish between different kinds of morphological effects. The steps involved in using an impedance-monitoring system for measurement of morphological modulation of cardiomyocytes may include: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding 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; allowing the cells to attach and spread; monitoring cardiomyocyte viability over time using the impedance-monitoring system at prespecified intervals of time for 12, 24, 48, 72 hours or longer; at certain times after cell seeding, treat the cell with agents that may cause morphology changes at one or more concentration; using the vehicle that the agent is dissolved in as a control; continuing to 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; and quantifying 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.

[0128] Once regular cardiomyocyte beating can be effectively monitored compounds may be added to the wells to assess whether or not beating frequency is altered, beating amplitude is altered and the like. Thus, compounds that adversely affect the normal cardiomyocyte beating are suspected of being cardiotoxic. However, cardioprotective agents can also be assessed by adding an agent suspected of being cardioprotective to the well and inducing a cardiotoxic response.

[0129] Accordingly, methods of identifying or screening for potential agents that modulate ES- derived cardiomyocyte beating or primary cardiomyocyte beating by monitoring and measuring the excitation-contraction coupling of cardiomyocytes upon treatment with pharmacological agents are also provided. The methods may include providing the system for monitoring impedance at millisecond resolution of excitable cells in vitro, adding ES cells or primary cardiomyocytes to two or more wells, adding a test agent suspected of modulating ES-derived or primary cardiomyocyte beating to a first well to provide a test well and either no agent or a control agent to a second well to form a control well, monitoring impedance of the, two or more wells at different or similar time intervals using millisecond time resolution at least in part, and optionally determining cell indices from impedance values, generating an impedance-based curve or optionally a cell index curve for each of the test well and control well, and comparing the impedance-based curves or optionally the cell index curves between the test well and the control well. If significantly different, the test agent is concluded to modulate cardiomyocyte function. In preferred embodiments, the impedance-based curves may be direct measurement of cardiomyocyte excitation-contraction coupling; however, the methods may also include long term monitoring of the viability and or morphology of the cardiomyocytes. The methods may show whether the test agent increases cardiomyocyte beating, which is indicative of increased heart rate, or whether the cardiomyocyte beating is slowed, which is indicative of lowered heart rate.

[0130] In another exemplary embodiment a method for screening for modulators of cardiomyocyte beating using an impedance-measurement system in vitro may include: providing a single-well or multi-well device that comprise micro-electrode arrays in well(s) of the device, which can be used for monitoring cell-substrate impedance; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding 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; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells; and adding the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency. Preferably, the step of monitoring impedance of beating is performed 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.

[0131] In another exemplary embodiment, a method of screening for modulators of cardiomyocyte beating using an impedance-based system in vitro includes seeding cardiomyocytes, in E-PLATES exactly as described in the previous exemplary embodiment; however, prior to addition of pharmacological agent, obtaining a baseline of the cardiomyocyte beating frequency using the impedance measurement system with millisecond time resolution; and adding the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency.

[0132] 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 some embodiments the present invention is directed to a 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 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 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 biologically active agent modulates cardiomyocyte function. Optionally, impedance-based curves or optionally cell index curves are used to calculate the compound dose-dependent changes in cardiomyocyte morphology and generate an EC-50 value for the potency of the compound.

[0133] 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 cardiomyocytes and cardiomyocyte morphology and differentiation and to screen for pharmacological agents which may modulate these processes and induce cardiotoxicity. In some embodiments, viability is monitored based on long term impedance monitoring of cardiomyocytes seeded in microelectronic plates (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 operably connected to an impedance analyzer, wherein the device includes at least one well optionally coated with fibronectin 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; In another aspect, the method includes providing a device for measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least two wells optionally coated with fibronectin 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 of the at least two wells over a period of time and optionally determining cell index from impedance values. 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 or alternatively the cell index of the treated well is significantly different than the impedance or cell index of the treated well.

[0134] In other preferred embodiments short term measurement using millisecond time resolution is combined with longer term impedance monitoring, such as over seconds, hours, days and the like. Certain cardiotoxic drugs can directly affect the viability of cardiomyocytes. Thus, long term impedance monitoring may be used to assess loss of viability of cardiomyocytes. An exemplary long term method to assess loss of viability includes: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding 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 seeding densities to the wells of the device; allowing the cells to attach and spread; monitoring 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; at certain time after cell seeding, treating the cell with a cytotoxic agent at one or more concentration; using the vehicle that the agent is dissolved in as a control; 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, quantify the extent of cardiotoxicity by normalizing the cell index values immediately prior to agent addition and determining 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.

[0135] 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 morphology operably connected to an optical measurement system, where the device includes at least two wells optionally coated with fibronectin 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 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 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.

[0136] One skilled in the art will recognize that any agent may be tested. The agent may be a biologically active agent.

Exemplary test agents include compounds, drugs, peptides, proteins, antibodies, antibody fragments, siRNA, shRNA, miRNA, cDNA, lipids and any combination thereof. 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 beats, comparison of these optionally derived parameters is made between the test well and the control well, and if significant differences exist, one may optionally conclude that the test agent modulates cardiomyocyte functions.

[0137] The study of cardiomyocyte function and modulation has particular utility in the biomedical arts. For example, the methods of the present invention may be used to assess whether a potential agent is cardiotoxic, cardioprotective and the like. If normal cardiomyocyte beating is altered, the result likely demonstrates the potential agent is cardiotoxic. Such information would permit effective screening prior to testing in live animals or in humans. Further, by coupling millisecond time resolution over extended time periods, the methods may assess short term and long term effects on cardiomyocytes. Since 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, the systems and methods are able to precisely measure and quantify these changes in cell morphology and shape. Thus the methods and systems have particular utility in the areas of biomedical research.

[0138] In some embodiments, potential agents are tested to assess whether they are cardioprotective, or protect against adverse affects. In such embodiments the agent suspected of being cardioprotective is added to the cardiomyocytes prior to, simultaneously or after adding a cardiotoxic agent or a cardiotoxic event. Thus, a cardioprotective agent may prevent abnormal shifts in beating cycle, which would naturally occur in the presence of a cardiotoxic agent. In further embodiments, cardiomyocytes having abnormal beating cycles are treated with potential agents to assess whether normal beating may be induced, such as through administration of a cardioprotective pharmacological agent.

[0139] Certain treatments can also affect the differentiative process of ES cells to cardiomyocytes which may involve specific morphological and adhesive changes. In another aspect of the present invention a method is provide to screen or potential agents that may modulate the morphology of ES- derived cardiomyocyte, adult stem cell-derived cardiomyocyte or primary cardiomyocyte or its differentiation.

[0140] Impedance-based curves and optionally cell index curves may be used to calculate 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. Significant difference between measurements or data of a test well and a control well support a finding that the biologically active agent within the test well modulates cardiomyocyte function.

E. Assessing the Developmental or Functional Consequence of Gene Knockout and Transgene Expression in Embryonic Stem Cell-Derived Cardiomyocytes

[0141] Embodiments of the present invention also include assessing the developmental consequence of genetically manipulated ES cells or their derived cardiomyocytes. 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 with millisecond time resolution can be used to evaluate the role of these genes in cardiac development and or function.

[0142] Steps involved in assessing the developmental and functional effect of gene knockout or transgene expression may include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene; provide the system for monitoring impedance of excitable cells having millisecond time resolution; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding the embryonic stem cells (ES cells) of mammalian origin at desired seeding densities into the wells of the device; allowing the cells to attach and spread; after a time period unique to ES-derived cardiomyocytes or primary cardiomyocytes, and monitoring cardiomyocyte beating using the impedance-monitoring system using millisecond kinetic readout to resolve the individual beat cycles of the cells. 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 impedance-based measurement 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. In alternative embodiments, 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 impedance-based measurement system with millisecond time resolution.

[0143] In other exemplary embodiments an impedance-based measurement system is used to evaluate the role of 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 may include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene; providing 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; optionally coat wells of the device with either fibronectin or other matrix proteins; seeding 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; allowing the cells to attach and spread and monitor the growth and viability of the cells using the impedance-based system; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring 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. 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. In related embodiments, 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.

[0144] In another exemplary embodiment, steps involved in assessing the developmental and functional effect of gene knockout or transgene expression using an impedance-based system in vitro are provided, which include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene; optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding either ES cells of mammalian origin or primary cardiomyocytes at specific seeding densities; allowing the cells to attach and spread; and after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-based system by using millisecond kinetic readout to resolve the individual beat cycles of the cells. 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 impedance-based 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. 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 impedance-based system.

[0145] In another aspect, the present invention is directed to method to establish an assay to assess the effect of gene knockout or transgene expression in ES cells differentiated to cardiomyocytes and functionally monitored by the impedance-based system. The method includes providing a device for measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least two wells; 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.

[0146] As above, in preferred embodiments, the time difference between two consecutive impedance measurement for a well is in the range of milliseconds (e.g., less than 500 milliseconds, less than 300 milliseconds, less than 100 milliseconds, less than 10 milliseconds, or less than about 1 millisecond or faster). The millisecond 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 using at least two time points for each beat cycle. In some embodiments more than two time points are resolved for each beating cycle.

EXAMPLES

Example 1

Impedance Monitoring of Attachment, Growth and Viability of Mouse ES Cell-Derived Cardiomyocytes

[0147] Since isolated primary cardiomyocytes, ES-derived cardiomyocytes and adult stem cell-derived cardiomyocytes can be maintained in culture, these cells provide a desirable model system to study cardiomyocytes as well as cardiomyocyte modulation. The present example demonstrates 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.

[0148] Mouse ES cells were seeded at a density ranging from 3000 cells to 50,000 cells per well in E-PLATES (Acea Biosciences, San Diego, CA) precoated with fibronectin. The attachment, growth and viability of the cells were monitored on RT-CES system measuring impedance signal in the form of cell index every 30 minute 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.

[0149] FIG. 3 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.

Example 2

Detection of Cardiotoxic Effects Using Real Time Impedance Monitoring

[0150] 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 (**FIG. 4**).

[0151] Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells per well in E-PLATES precoated with fibronectin. The attachment and growth of 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 **FIG. 4A** 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 **FIG. 4B**, half maximal activity or IC-50 value of 1.48 μ M was derived for the compound.

Example 3

Real time Monitoring of Mouse ES Cell-Derived Cardiomyocytes and Inducing Hypertrophy

[0152] 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 isoproterenol, a β 2 adrenergic receptor agonist known to induce hypertrophy (**FIG. 5A**).

[0153] Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells per well in E-PLATES 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 isoproterenol. According to **FIG. 5A**, isoproterenol 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 isoproterenol-induced morphological changes in mouse ES-derived cardiomyocytes, the normalized cell indices were plotted against the log of the corresponding isoproterenol concentrations (**FIG. 5B**). From the sigmoidal curve generated a half maximal activity or IC-50 value of 3.1 nM was derived for the compound.

Example 4

Real Time Impedance Monitoring with Millisecond Resolution of Beating Cardiomyocytes

[0154] As an example, we describe here the use of an improved impedance-based system with millisecond time resolution to measure and monitor the beating of cardiomyocytes using fast kinetic software.

[0155] Mouse ES cells were seeded at a density of between 3,000 to 50,000 cells per well in E-PLATES precoated with fibronectin. The attachment and growth of the cells were monitored on RT-CES system. **FIG. 6** 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 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 up to 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. We now understand the origins of such "small-spikes" and interpret that such spikes in the impedance or cell index readout are associated with the 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.

[0156] 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 milliseconds) 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 **FIGS. 3** and **6**, where the background measurement is performed using cell culture media). The software was used to measure cardiomyocyte beating at distinct stages throughout its attachment and growth phases (**FIG. 7**). 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 (**FIG. 7A**). For plot, impedance readout has been converted into dimensionless cell indices. **FIGS. 7B** and **7C** 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.

[0157] 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.

[0158] 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 to determine such "rise" and "decay" portions of the curves and then counts a peak.

[0159] Another method to derive or count the beating rates of cardiomyocytes 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 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. 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. **FIG. 8A** and **FIG. 8B** shows a pair of cell index curves and the corresponding beat rate based the above described Fourier transform. In **FIG. 8B**, the cell index curves last from time zero to time 42 seconds. In **FIG. 8B**, the beating frequency for the traces in **FIG. 8A** 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 **FIG. 8B**, 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 **FIG. 8B** starts from about 20 seconds to 42 seconds.

[0160] 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 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. **FIG. 9A** 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 **FIGS. 9C** and **9D**, where the time resolution between two adjacent points is 40 milliseconds. In other words, a second in **FIGS. 9C** and **9D** is equivalent to 40-milliseconds.

[0161] 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. **FIG. 9B** 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 μM . 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 curves.

[0162] 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 starting points of the peak can be used for the length of time between the beats.

[0163] 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.

Example 5

Identifying Modulators of Cardiac Function Using Real Time Impedance Monitoring with Millisecond Resolution

[0164] Similar to Example 4, the millisecond kinetic readout requires that the impedance measurement system can provide impedance measurement data at millisecond time resolution. In other words, the time difference between two consecutive impedance measurement for a well shall be in the range of milliseconds (e.g., less than 500 millisecond, less than 300 millisecond, less than 100 millisecond, less than 10 millisecond, or less than 1 millisecond or faster). The millisecond 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.

[0165] In order to demonstrate the utility of the millisecond kinetic impedance-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 differentiated into beating cardiomyocytes, as described in Section 4. 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 final concentration of 333 nM and cardiomyocyte beating was monitored for 10 minutes (FIG. 10A). 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 cardiomyocyte beating from ~ 65 beats/min to 115-135 beats/min (FIG. 10B 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 FIGS. 9C and 9D, the time resolution between two adjacent points in FIGS. 10A and 10B is 40 milliseconds. In other words, a second in FIGS. 10A and 10B is equivalent to 40 millisecond.

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
Isoproterenol	b2 Adrenergic receptor agonist	4.4 uM	From ~ 65 to 115-136	From 0.19 to 0.16	Beating rate increased

[0166] To further demonstrate the capabilities of the improved 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 RT-CES system. Approximately, 72 hours after cell seeding the improved, impedance-measurement system with millisecond 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 the indicated drug and dose shown in FIG. 11. Similar to the cell index plot shown in FIGS. 9C and 9D, the time resolution between two adjacent points in all the figures in FIG. 9 is 40 milliseconds. In other words, a second in FIG. 9 is equivalent to 40-milliseconds.

[0167] 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 FIG. 12 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 impedance-measurement system with millisecond time resolution 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 FIGS. 9C and 9D, the time resolution between two adjacent points in all the figures in FIG. 10 is 40 milliseconds. In other words, a second in a

figure in FIG. 10 is equivalent to 40-millisecond.

Table II.

Compound	Mechanism	Concentration	Beat Rate	Amplitude	Pattern Change
Astemizole	antihistamine	400 nM	0	<0.01	From 68-72 bpm to irregular being to beating stopped
Terfenadine	antihistamine	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		13.3 uM	From ~ 80 to ~ 160	From 0.27 to 0.07	Much faster, pattern changed
E4031	K channel inhibitor	120 nM	From 80 to 160	From 0.09 to 0.03	Pattern changed, much faster
DDT	Pesticide	8 uM	From 80 to 140 then to ~ 80	From 0.25 to 0.02	Initially become faster, later irregular beating
PCB	Organic toxicant	8uM	From ~ 65 to 0	From 0.2 to 0	Beating stopped
Endosulfan	insecticide	8uM	From ~ 75 to 0	From 0.2 to 0	Beating stopped

[0168] To further demonstrate the capabilities of the fast impedance-measurement-system-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 with millisecond 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. **FIG. 13** 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 **FIGS. 9C** and **9D**, the time resolution between two adjacent points in all the figures in **FIG. 11** is 40 milliseconds. In other words, a second in **FIG. 13** is equivalent to 40 milliseconds.

Example 6

Modulation of Cardiomyocyte Beating In Response to Haloperidol Treatment Using Millisecond Time Resolution

[0169] To further demonstrate the capabilities of the improved impedance measurement system for monitoring of cardiomyocyte beating in detecting drugs which may adversely affect heart function, 500nM Haloperidol was tested to demonstrate its time-dependent effects on cardiomyocytes. Approximately, 72 hours after cell seeding the improved impedance system was used to establish a baseline reading of cardiomyocyte beating for each well for about 16 seconds (**FIG. 14A**). Note that in **FIG. 14A**, each minute showed on the x-axis of **FIG. 14A** corresponds to one second in real measurement. Subsequently, the cells in the well treated with 500 nM Haloperidol. **FIGS. 14B, C, D, E, F, G** and **H** show the impedance-based monitoring of beating of the cardiomyocytes at time points of first 30 seconds, about 1 minute, about 2 minutes, about 4 minutes, about 5.5 minutes, about 1 hour and about 21 hours after the compound treatment. In **FIGS. 14B** through **14H**, each minute showed on the x-axis corresponds to one second in real measurement. It is clear that within 1 hr of compound treatment, the cardiomyocytes exhibit significant, time-dependent change in their impedance responses. Even at ~21 hrs after compound treatment, the impedance pattern did not restore and the beating frequency (50 ~ 60 beats per minute) appeared to become about half of that before compound treatment (90 ~ 100 beats per minute before treatment). Yet the amplitude of the impedance response spikes nearly doubled compared with that before compound treatment. **FIG. 15** and **FIG. 16** show the analysis results of the impedance beating pattern of **FIGS. 14** in terms of the beating frequency (beats per minute) and the beating magnitude. For **FIG. 15** and **FIG. 16**, each unit (1) on the x-axis corresponds to 0.016 second (16 milliseconds) in real measurement.

Example 7

Modulation of Cardiomyocyte Beating In Response to Erythromycin Treatment Using Millisecond Time Resolution

[0170] To further demonstrate the capabilities of the improved impedance measurement system for monitoring of cardiomyocyte beating in detecting drugs which may adversely affect heart function, 300 uM erythromycin was tested to demonstrate its time-dependent effects on cardiomyocytes. Approximately, 72 hours after cell seeding the improved impedance system was used to establish a baseline reading of cardiomyocyte beating for each well for about 15 seconds (**FIG. 17A**). Note that in **FIG. 17A**, each minute showed on the x-axis of **FIG. 17A** corresponds to one second in real measurement. Subsequently, the cells in the well treated with 300 uM erythromycin. **FIGS. 17B-I** show the impedance-based monitoring of beating of the cardiomyocytes at time points of first 30 seconds, about 1 minute, about 2 minutes, about 4 minutes, about 5.5 minutes, about 1 hour, about 3hr and about 21 hours after the compound treatment. In **FIGS. 17B-H**, each minute showed on the x-axis corresponds to one second in real measurement. It is clear that within 3 hr of compound treatment, the cardiomyocytes exhibit significant, time-dependent change in their impedance responses. For example, at about 1 hr after the treatment, the frequency of the impedance-based beating spikes is much higher than that for the cardiomyocytes before treatment, yet the magnitude is much smaller. On the other hand, at about 3 hr after treatment, the impedance-based beating pattern becomes rather irregular whilst the magnitude of the beating pattern has somewhat restored. Still, even at ~21 hrs after compound treatment, the impedance pattern did not restore and the beating frequency appeared to about 70% (at 50 ~ 60 beats per minute) of that before compound treatment (at 80 ~ 90 beats per minute). Yet the amplitude of the impedance response spikes increased by about 50% compared with that before compound treatment. **FIG. 18** and **FIG. 19** show the analysis results of the impedance beating pattern of **FIGS. 17** in terms of the beating frequency (beats per minute) and the beating magnitude. For **FIG. 18** and **FIG. 19**, each unit (1) on the x-axis corresponds to 0.016 second (16 milli-seconds) in real measurement.

Claims

1. A system for monitoring impedance of excitable cells in vitro, comprising:

a) a device for monitoring cell-substrate impedance, comprising

i) a nonconductive substrate (101);

ii) one or more electrode arrays (102) fabricated on said substrate (101);

iii) one or more wells on said substrate (101), wherein each of said one or more arrays (102) is associated with one of said one or more wells, wherein each electrode array (102) is individually addressable, further wherein said substrate (101) has a surface suitable for cell attachment, wherein said cell attachment on said substrate (101) can result in a detectable change in impedance between electrodes within each electrode array (102);

b) an impedance analyzer capable of impedance measurement at millisecond time resolution comprising at least two consecutive impedance measurements less than 20 milliseconds apart;

c) electronic circuitry that can engage said device and selectively connect said two or more electrode arrays of said device to said impedance analyzer; and

d) a software program that controls said electronic circuitry and records and analyzes data obtained from said impedance analyzer.

2. A system as claimed in claim 1, wherein each of said electrode arrays comprises two electrode structures having substantially a same surface area; optionally wherein

(a) said electrode structures are each constructed from a material selected from the group consisting of metal, gold, platinum, titanium and chromium; or

(b) said electrode structures have a width between 20 microns and 500 microns; or

(c) a gap between electrode structures within each electrode array is between 5 microns and 100 microns.

3. A system as claimed in claim 1, wherein each electrode array is organized in a configuration selected from the group consisting of interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal, and castellated.

4. A system as claimed in claim 1, wherein said millisecond time resolution allows for impedance measurements at more than two time points within each excitation cycle of excitable cells introduced to wells; optionally

(a) wherein said excitation cycle is a cell beating cycle; preferably

wherein said excitable cells are cardiomyocytes; or

(b) wherein said excitable cells are excitation-contraction coupling cells.

5. A system as claimed in claim 1, wherein said at least two consecutive impedance measurements are less than 10 milliseconds apart.

6. A system as claimed in any preceding claim, wherein said one or more wells is selected from a group of 6 wells, 8 wells, 16 wells, 32 wells, 96 wells and 384 wells.

7. A method for assessing excitable cells in vitro using a system according to any one of claims 1 to 6, the method comprising

a) adding excitable cells to one or more wells of the device comprised in the system;

b) monitoring impedance of said one or more wells using millisecond time resolution; and

c) resolving individual cycles of said excitable cells.

8. A method as claimed in claim 7, wherein said excitable cells are cells undergo excitation-contraction coupling; preferably wherein said excitable cells are cardiomyocytes.

9. A method as claimed in claim 7, wherein the step of monitoring impedance comprises performing impedance measurements at millisecond time resolution simultaneously across all wells.

10. A method as claimed in claim 7 for determining whether a compound modulates a beating cycle of an excitable cell, wherein the one or more wells is at least two wells, the method further comprising:

adding a compound suspected of modulation of the beating cycle to a first of said at least two wells to provide a test well, wherein a second well lacking compound is provided as a control well, wherein the step of resolving individual cycles of said excitable cells comprises resolving the beating cycles of both test well and control well; and
comparing said beating cycles between said test well and control well, wherein a difference in beating cycles indicates said compound modulates the beating cycle of the excitable cell.

11. A method as claimed in claim 10, wherein

(a) the excitable cells are cardiomyocytes or embryonic stem cell derived cardiomyocytes;
and/or

(b) wherein said beating cycles are compared to identify

(i) changes in beating amplitude; or

(ii) changes in beating frequency; preferably

wherein said changes in beating frequency are regular changes or irregular changes.

12. A method as claimed in claim 10 or claim 11, further comprising determining a cell index for each of said test well and control well, wherein the step of comparing said beating cycles comprises comparing said cell indexes between test well and control well.

13. A method of characterizing rhythmic beating of a cardiomyocyte using a system according to any of claims 1 to 6, the method comprising:

- a) adding excitable cells to one or more wells of the device comprised in the system;
- b) monitoring impedance of said one or more wells in millisecond time resolution; and
- c) determining a plurality of beating cycle peaks in a time unit; and
- d) comparing amplitude or frequency of said peaks over said time unit.

14. A method as claimed in claim 13, wherein

- (a) the step of determining a plurality of beating cycle peaks comprises determining a rise in impedance and a decay in impedance and extrapolating a peak from each rise and decay; or
- (b) beating cycle peaks are determined by identifying impedance data points at which the first order derivative to time is zero or close to zero.

15. A method as claimed in claim 13, further comprising

- (a) determining a cell index for each of said one or more wells, wherein the step of determining a plurality of beating cycle peaks comprises for each cycle determining a rise in cell index, a decay in cell index and extrapolating each peak from each rise and decay; and/or
- (b) monitoring impedance of said one or more wells over seconds, minutes or hours, optionally wherein said monitoring of impedance in millisecond time resolution is performed within said monitoring over seconds, minutes or hours.

16. A method of assessing genetic manipulation of an embryonic stem cell using a system according to any of claims 1 to 6, wherein the one or more wells are at least two wells, the method comprising:

- a) mutating embryonic stem cells to comprise a genetic mutation;
- b) adding said mutated embryonic stem cells to a first well of said at least two wells to form a test well and control embryonic stem cells to a second of said at least two wells to form a control well;
- c) monitoring impedance of said at least two wells in millisecond time resolution; and
- d) resolving the beating cycles of both test well and control well, if any; and

e) comparing said beating cycles between said test well and control well, wherein a difference in beating cycles indicates said genetic mutation modulates embryonic stem cell differentiation.

17. A method as claimed in claim 16, wherein

- (a) said embryonic stem cell differentiation is differentiation into a cardiomyocyte; or
- (b) said genetic mutation is selected from the group consisting of a genetic knock out, a knock down and an inserted transgene.

18. The method as claimed in claim 7, the method further comprising:

monitoring impedance of said one or more wells over a first time period, which is characterized as a longer time period comprising seconds, minutes or hours, further wherein the step of monitoring impedance of said one or more wells using millisecond time resolution is performed over a second time period, further wherein the step of resolving individual cycles of said excitable cells is performed from monitoring impedance over said second time period; and
determining a characteristic selected from the group consisting of cell attachment, cell growth and cell viability from monitoring over said first time period;
wherein the step of resolving individual cycles of said excitable cells is performed from said monitoring impedance over said second time period.

19. A method as claimed in claim 18, wherein monitoring impedance over said second time period is performed at regular or irregular time intervals within monitoring impedance over said first time period.

Patentansprüche

1. System zur Überwachung der Impedanz erregbarer Zellen in vitro, wobei das System folgendes umfasst:

a) eine Vorrichtung zur Überwachung der Zellen-Substrat-Impedanz, wobei die Vorrichtung folgendes umfasst<:

- i) eine nicht leitfähiges Substrat (101);
- ii) eine oder mehrere Elektrodenanordnungen (102), die auf dem genannten Substrat (101) ausgebildet sind;
- iii) eine oder mehrere Schalen auf dem genannten Substrat (101), wobei jede der genannten einen oder mehreren Anordnungen (102) einer der genannten einen oder mehreren Schalen zugeordnet ist, wobei jede Elektrodenanordnung (101) einzeln adressierbar ist, wobei ferner das genannte Substrat (101) eine Oberfläche aufweist, die sich für die Zellenanhaftung eignet, wobei die genannte Zellenanhaftung an dem genannten Substrat (101) zu einer nachweisbaren Veränderung der Impedanz zwischen Elektroden in jeder Elektrodenanordnung (102) führen kann;

b) einen Impedanzanalysator, der eine Impedanzmessung mit einer zeitlichen Auflösung im Millisekundenbereich vornehmen kann, umfassend mindestens zwei aufeinanderfolgende Impedanzmessungen, die weniger als 20 Millisekunden auseinander liegen;

c) eine elektronische Schaltkreisanordnung, die mit der genannten Vorrichtung eingreifen kann und selektiv die genannten zwei oder mehreren Elektrodenanordnungen der genannten Vorrichtung mit dem genannten Impedanzanalysator verbinden kann; und

d) ein Softwareprogramm, das die genannte elektronische Schaltkreisanordnung steuert und von dem genannten Impedanzanalysator erhaltene Daten aufzeichnet und analysiert.

2. System nach Anspruch 1, wobei jede der genannten Elektrodenanordnungen zwei Elektrodenstrukturen umfasst, die im Wesentlichen den gleichen Oberflächenbereich aufweisen, wobei optional:

- (a) die genannten Elektrodenstrukturen jeweils aus einem Material bestehen, das ausgewählt ist aus der Gruppe bestehend aus Metall, Gold, Platin, Titan und Chrom; oder
- (b) die genannten Elektrodenstrukturen eine Breite zwischen 20 Mikron und 500 Mikron aufweisen; oder
- (c) ein Abstand zwischen Elektrodenstrukturen in jeder Elektrodenanordnung zwischen 5 Mikron und 100 Mikron liegt.

3. System nach Anspruch 1, wobei jede Elektrodenanordnung in einer Konfiguration organisiert ist, die ausgewählt ist aus der Gruppe bestehend aus verschachtelt, Kreis auf Linie, Raute auf Linie, konzentrisch, sinuskurvenförmig und mit Türmchen versehen.

4. System nach Anspruch 1, wobei die genannte zeitliche Auflösung im Millisekundenbereich Messungen zu mehr als zwei Zeitpunkten innerhalb jedes Erregungszyklus erregbarer Zellen ermöglicht, die in Schalen gegeben werden; wobei optional:

- (a) der genannte Erregungszyklus ein Zellenschlagzyklus ist; wobei die genannten erregbaren Zellen vorzugsweise Kardiomyozyten sind; oder wobei
- (b) die erregbaren Zellen Erregungs-Kontraktions-Kopplungszellen sind.

5. System nach Anspruch 1, wobei die genannten mindestens zwei aufeinanderfolgenden Impedanzmessungen weniger als 10 Millisekunden auseinander liegen.

6. System nach einem der vorstehenden Ansprüche, wobei die genannte eine oder die mehreren Schalen ausgewählt sind aus einer Gruppe von sechs Schalen, acht Schalen, 16 Schalen, 32, Schalen, 96 Schalen und 384 Schalen.

7. Verfahren zur Bewertung erregbarer Zellen in vitro unter Verwendung eines Systems nach einem der vorstehenden Ansprüche 1 bis 6, wobei das Verfahren folgendes umfasst:

- a) Hinzufügen erregbarer Zellen in eine oder mehrere Schalen der Vorrichtung, die das System umfasst;
- b) Überwachen der Impedanz der genannten einen oder mehreren Schalen unter Verwendung einer zeitlichen Auflösung im Millisekundenbereich; und
- c) Auflösen einzelner Zyklen der genannten erregbaren Zellen.

8. Verfahren nach Anspruch 7, wobei die genannten erregbaren Zellen Zellen sind, die einer Erregungs-Kontraktions-Kopplung unterzogen werden; wobei die genannten erregbaren Zellen Kardiomyozyten sind.

9. Verfahren nach Anspruch 7, wobei die Überwachung der Impedanz das Durchführen von Impedanzmessungen mit einer zeitlichen Auflösung im Millisekundenbereich gleichzeitig für alle Schalen umfasst.

10. Verfahren nach Anspruch 7 zum Bestimmen, ob eine Verbindung einen Schlagzyklus einer erregbaren Zelle moduliert, wobei die eine oder mehreren Schalen mindestens zwei Schalen sind, wobei das Verfahren ferner folgendes umfasst:

Hinzufügen einer Verbindung, für die angenommen wird, dass sie den Schlagzyklus einer ersten der genannten mindestens zwei Schalen moduliert, um eine Testschale vorzusehen, wobei eine zweite Schale, die keine Verbindung aufweist, als Kontrollschale vorgesehen ist, wobei der Schritt des Auflöser einzelner Zyklen der genannten erregbaren Zellen das Auflösen der Schlagzyklen sowohl der Testschale als auch der Kontrollschale umfasst; und
Vergleichen der genannten Schlagzyklen zwischen der genannten Testschale und der Kontrollschale, wobei eine Differenz der Schlagzyklen anzeigt, dass die genannte Verbindung den Schlagzyklus der erregbaren Zelle moduliert.

11. Verfahren nach Anspruch 10, wobei

- (a) die erregbaren Zellen Kardiomyozyten oder aus embryonalen Stammzellen abgeleitete Kardiomyozyten sind; und/oder
- (b) wobei die Schlagzyklen verglichen werden, um folgendes zu identifizieren:
 - (i) Veränderungen der Schlagamplitude; oder
 - (ii) Veränderungen der Schlagfrequenz; wobei die genannten Veränderungen der Schlagfrequenz vorzugsweise regelmäßige Veränderungen oder unregelmäßige Veränderungen sind.

12. Verfahren nach Anspruch 10 oder Anspruch 11, ferner umfassend das Bestimmen eines Zellenindex für jede der genannten Testschale und Kontrollschale, wobei der Schritt des Vergleichens der genannten Schlagzyklen das Vergleichen der genannten Zellenindizes zwischen der Testschale und der Kontrollschale umfasst.

13. Verfahren zur Charakterisierung des rhythmischen Schlagens eines Kardiomyozyts unter Verwendung eines Systems nach einem der Ansprüche 1 bis 6, wobei das Verfahren folgendes umfasst:

- a) Hinzufügen erregbarer Zellen in eine oder mehrere Schalen der Vorrichtung, die das System umfasst;
- b) Überwachen der Impedanz der genannten einen oder mehreren Schalen mit einer zeitlichen Auflösung im Millisekundenbereich;
- c) Bestimmen einer Mehrzahl von Schlagzyklusspitzen in einer Zeiteinheit; und
- d) Vergleichen der Amplitude oder Frequenz der genannten Spitzen über die genannte Zeiteinheit.

14. Verfahren nach Anspruch 13, wobei:

- (a) der Schritt des Bestimmens einer Mehrzahl von Schlagzyklusspitzen das Bestimmen eines Anstiegs der Impedanz und eines Abfallens der Impedanz umfasst sowie das Extrapolieren einer Spitze aus jedem Anstieg und jedem Abfall; oder
- (b) Schlagzyklusspitzen bestimmt werden durch Identifizieren von Impedanzdatenpunkten, bei denen die erste Ableitung auf die Zeit Null oder nahe Null ist.

15. Verfahren nach Anspruch 13, wobei dieses ferner folgendes umfasst:

- (a) Bestimmen eines Zellenindex für jede der genannten einen oder mehreren Schalen, wobei der Schritt des Bestimmens einer Mehrzahl von Schlagzyklusspitzen für jeden Zyklus das Bestimmen eines Anstiegs in einem Zellenindex, eines Abfalls in einem Zellenindex und das Extrapolieren jeder Spitze aus jedem Anstieg und Abfall umfasst; und/oder
- (b) Überwachen der Impedanz der genannten einen oder mehreren Schalen über Sekunden, Minuten oder Stunden, wobei das genannte Überwachen der Impedanz mit einer zeitlichen Auflösung im Millisekundenbereich im Rahmen des genannten Überwachens über Sekunden, Minuten oder Stunden ausgeführt wird.

16. Verfahren zur Bewertung der genetischen Manipulation einer embryonalen Stammzelle unter Verwendung eines Systems nach einem der Ansprüche 1 bis 6, wobei die eine oder mehreren Schalen wenigstens zwei Schalen sind, wobei das Verfahren folgendes umfasst:

- a) Mutieren embryonaler Stammzellen, um eine genetische Mutation zu umfassen;
- b) Hinzufügen der genannten embryonalen Stammzellen in eine erste Schale der genannten wenigstens zwei Schalen, um eine Testschale zu bilden und um embryonale Stammzellen in eine zweite der genannten wenigstens zwei Schalen zu kontrollieren, um eine Kontrollschale zu bilden;
- c) Überwachen der Impedanz der genannten wenigstens zwei Schalen in zeitlicher Auflösung im Millisekundenbereich; und
- d) Auflösen der Schlagzyklen sowohl der Testschale als auch der Kontrollschale, wobei eine Differenz der Taktzyklen anzeigt, dass die genannte genetische Mutation eine embryonale Stammzellendifferenzierung moduliert.

17. Verfahren nach Anspruch 16, wobei:

- (a) die genannte embryonale Stammzellendifferenzierung eine Differenzierung in ein Kardiomyozyt ist; oder
- (b) die genannte genetische Mutation ausgewählt ist aus der Gruppe bestehend aus einem genetischen Knock-out, einem Knock-Down und einem eingefügten Transgen.

18. Verfahren nach Anspruch 7, wobei das Verfahren ferner folgendes umfasst:

- Überwachen der Impedanz der genannten einen oder mehreren Schalen über einen ersten Zeitraum, der als ein längerer Zeitraum gekennzeichnet ist, der Sekunden, Minuten oder Stunden umfasst, wobei der Schritt des Überwachens der Impedanz der genannten einen oder mehreren Schalen unter Verwendung einer zeitlichen Auflösung im Millisekundenbereich ausgeführt wird über einen zweiten Zeitraum, wobei der Schritt des AuflöSENS einzelner Zyklen der genannten erregbaren Zellen ferner ausgeführt wird von der Überwachung der Impedanz über den genannten zweiten Zeitraum; und
- Bestimmen einer Eigenschaft, die ausgewählt ist aus der Gruppe bestehend aus Zellenanhaftung, Zellenwachstum und Zellenlebensfähigkeit über den genannten ersten Zeitraum; wobei der Schritt des AuflöSENS einzelner Zyklen der genannten erregbaren Zellen ausgeführt wird von der

Überwachung der Impedanz über den genannten zweiten Zeitraum.

19. Verfahren nach Anspruch 18, wobei das Überwachen der Impedanz über den genannten zweiten Zeitraum in regelmäßigen oder unregelmäßigen Zeitintervallen innerhalb der Überwachung der Impedanz über den genannten ersten Zeitraum ausgeführt wird.

Revendications

1. Système de surveillance de l'impédance de cellules pouvant être excitées in vitro, comprenant :

a) un dispositif de surveillance de l'impédance de substrat de cellules, comprenant

i) un substrat non conducteur (101) ;

ii) une ou plusieurs matrices (102) d'électrodes fabriquées sur ledit substrat (101) ;

iii) un ou plusieurs puits sur ledit substrat (101), chacune desdites une ou plusieurs matrices (102) étant associée à l'un desdits un ou plusieurs puits, chaque matrice (102) d'électrodes étant adressable individuellement, en outre ledit substrat (101) ayant une surface apte à la fixation de cellules, ladite fixation de cellules sur ledit substrat (101) pouvant se traduire par un changement détectable de l'impédance entre des électrodes dans chaque matrice (102) d'électrodes ;

b) un analyseur d'impédance apte à mesurer une impédance à une résolution dans le temps de l'ordre de la milliseconde comprenant au moins deux mesures d'impédance consécutives à moins de 20 millisecondes d'intervalle ;

c) un circuit électronique qui peut venir en contact avec ledit dispositif et connecter de manière sélective lesdites deux ou plusieurs matrices d'électrodes dudit dispositif audit analyseur d'impédance ; et

d) un programme logiciel qui commande ledit circuit électronique et enregistre et analyse les données obtenues dudit analyseur d'impédance.

2. Système selon la revendication 1, chacune desdites matrices d'électrodes comprenant deux structures d'électrode ayant sensiblement une même surface ; éventuellement

(a) lesdites structures d'électrodes étant chacune construites en un matériau choisi dans le groupe constitué de métal, d'or, de platine, de titane et de chrome ; ou

(b) lesdites structures d'électrodes ayant une largeur comprise entre 20 et 500 microns ; ou

(c) un écart entre les structures d'électrodes dans chaque matrice d'électrodes étant compris entre 5 et 100 microns.

3. Système selon la revendication 1, chaque matrice d'électrodes étant organisée en une configuration choisie parmi inter-digitée, cercle-sur-ligne, losange-sur-ligne, concentrique, sinusoïdale, et crénelée.

4. Système selon la revendication 1, ladite résolution dans le temps de l'ordre de la milliseconde permettant des mesures d'impédance à plus de deux points de temps dans chaque cycle d'excitation de cellules pouvant être excitées introduites dans des puits ; éventuellement

(a) ledit cycle d'excitation étant un cycle de battement de cellule ; de préférence lesdites cellules pouvant être excitées étant des cardiomyocytes ; ou

(b) lesdites cellules pouvant être excitées étant des cellules de couplage excitation-contraction.

5. Système selon la revendication 1, lesdites au moins deux mesures d'impédance consécutives étant à moins de 10 millisecondes d'intervalle.

6. Système selon l'une quelconque des revendications précédentes, lesdits un ou plusieurs puits étant choisis dans un groupe de 6 puits, 8 puits, 16 puits, 32 puits, 96 puits et 384 puits.

7. Procédé d'évaluation de cellules pouvant être excitées in vitro à l'aide d'un système selon l'une quelconque des revendications 1 à 6, le procédé comprenant les étapes consistant à :

- a) ajouter des cellules pouvant être excitées à un ou plusieurs puits du dispositif compris dans le système ;
- b) surveiller l'impédance desdits un ou plusieurs puits à l'aide d'une résolution dans le temps de l'ordre de la milliseconde ; et
- c) résoudre des cycles individuels desdites cellules pouvant être excitées.

8. Procédé selon la revendication 7, lesdites cellules pouvant être excitées étant des cellules subissant un couplage excitation-contraction ; de préférence lesdites cellules pouvant être excitées étant des cardiomyocytes.

9. Procédé selon la revendication 7, la surveillance de l'impédance comprenant l'étape consistant à effectuer des mesures d'impédance à une résolution dans le temps de l'ordre de la milliseconde simultanément sur tous les puits.

10. Procédé selon la revendication 7 destiné à déterminer si un composé module un cycle de battement d'une cellule pouvant être excitée, les un ou plusieurs puits étant au moins deux puits, le procédé comprenant en outre les étapes consistant à :

ajouter un composé soupçonné de modulation du cycle de battement à un premier puits desdits au moins deux puits pour fournir un puits d'essai, un second puits sans composé étant utilisé comme puits de contrôle, la résolution de cycles individuels desdites cellules pouvant être excitées comprenant l'étape consistant à résoudre les cycles de battement des puits d'essai et de contrôle ; et

comparer lesdits cycles de battement entre ledit puits d'essai et ledit puits de contrôle, une différence des cycles de battement indiquant que ledit composé module le cycle de battement de la cellule pouvant être excitée.

11. Procédé selon la revendication 10,

(a) les cellules pouvant être excitées étant des cardiomyocytes ou des cardiomyocytes dérivées de cellules souches embryonnaires ; et/ou

(b) lesdits cycles de battement étant comparés pour identifier

(i) des changements dans l'amplitude de battement ; ou

(ii) des changements dans la fréquence de battement ; de préférence

lesdits changements dans la fréquence de battement étant des changements réguliers ou des changements irréguliers.

12. Procédé selon la revendication 10 ou 11, comprenant en outre l'étape consistant à déterminer un indice de cellule pour chacun desdits puits d'essai et puits de contrôle et, la comparaison desdits cycles de battement comprenant l'étape consistant à comparer lesdits indices de cellule entre le puits d'essai et le puits de contrôle.

13. Procédé de caractérisation d'un battement rythmique d'une cardiomyocyte à l'aide d'un système selon l'une quelconque des revendications 1 à 6, le procédé comprenant les étapes consistant à :

a) ajouter des cellules pouvant être excitées à un ou plusieurs puits du dispositif compris dans le système ;

b) surveiller l'impédance desdits un ou plusieurs puits dans une résolution dans le temps de l'ordre de la milliseconde ; et

c) déterminer une pluralité de sommets de cycle de battement dans une unité de temps ; et

d) comparer l'amplitude ou la fréquence desdits sommets sur ladite unité de temps.

14. Procédé selon la revendication 13,

(a) la détermination d'une pluralité de sommets de cycle de battement comprenant les étapes consistant à déterminer une augmentation de l'impédance et une diminution de l'impédance et à extrapoler un sommet à partir de chaque augmentation et diminution ; ou

(b) les sommets de cycle de battement étant déterminés en identifiant des points de données d'impédance auxquels la dérivée de premier ordre par rapport au temps est zéro ou proche de zéro.

15. Procédé selon la revendication 13, comprenant en outre les étapes consistant à :

(a) déterminer un indice de cellule pour chacun desdits un ou plusieurs puits, la détermination d'une pluralité

de sommets de cycle de battement comprenant, pour chaque cycle, les étapes consistant à déterminer une augmentation de l'indice de cellule, une diminution de l'indice de cellule et à extrapoler chaque sommet à partir de chaque augmentation et diminution ; et/ou

(b) surveiller l'impédance desdits un ou plusieurs puits sur plusieurs secondes, minutes ou heures, éventuellement ladite surveillance de l'impédance dans une résolution dans le temps de l'ordre de la milliseconde étant réalisée dans ladite surveillance sur plusieurs secondes, minutes ou heures.

16. Procédé d'évaluation de la manipulation génétique d'une cellule souche embryonnaire à l'aide d'un système selon l'une quelconque des revendications 1 à 6, le ou les plusieurs puits étant au moins deux puits, le procédé comprenant les étapes consistant à :

a) muter des cellules souches embryonnaires pour comprendre une mutation génétique ;

b) ajouter lesdites cellules souches embryonnaires mutées à un premier puits desdits au moins deux puits pour former un puits d'essai et contrôler lesdites cellules souches embryonnaires à un second desdits au moins deux puits pour former un puits de contrôle ;

c) surveiller l'impédance desdits au moins deux puits dans une résolution dans le temps de l'ordre de la milliseconde ; et

d) résoudre les cycles de battement du puits d'essai et du puits de contrôle, le cas échéant ; et

e) comparer lesdits cycles de battement entre ledit puits d'essai et ledit puits de contrôle, une différence des cycles de battement indiquant que ladite mutation génétique module la différenciation de cellule souche embryonnaire.

17. Procédé selon la revendication 16,

(a) ladite différenciation de cellules souches embryonnaires étant la différenciation en une cardiomyocytes ; ou
(b) ladite mutation génétique étant choisie dans le groupe constitué d'un knockout génétique, d'un knock-down et d'un transgène inséré.

18. Procédé selon la revendication 7, le procédé comprenant en outre les étapes consistant à :

surveiller l'impédance desdits un ou plusieurs puits sur une première période de temps, qui est caractérisée comme étant une période de temps plus longue comprenant des secondes, minutes ou heures, la surveillance de l'impédance desdits un ou plusieurs puits à l'aide d'une résolution dans le temps de l'ordre de la milliseconde étant effectuée sur une seconde période de temps, la résolution de cycles individuels desdites cellules pouvant être excitées étant effectuée à partir de la surveillance d'impédance sur ladite seconde période de temps ; et déterminer une caractéristique choisie dans le groupe constitué d'une fixation de cellule, d'une croissance de cellule et d'une viabilité de cellule à partir de la surveillance sur ladite première période de temps ; la résolution de cycles individuels desdites cellules pouvant être excitées étant effectuée à partir de ladite surveillance de l'impédance sur ladite seconde période de temps.

19. Procédé selon la revendication 18, la surveillance de l'impédance sur ladite seconde période de temps étant effectuée à des intervalles de temps réguliers ou irréguliers pendant la surveillance de l'impédance sur ladite première période de temps.

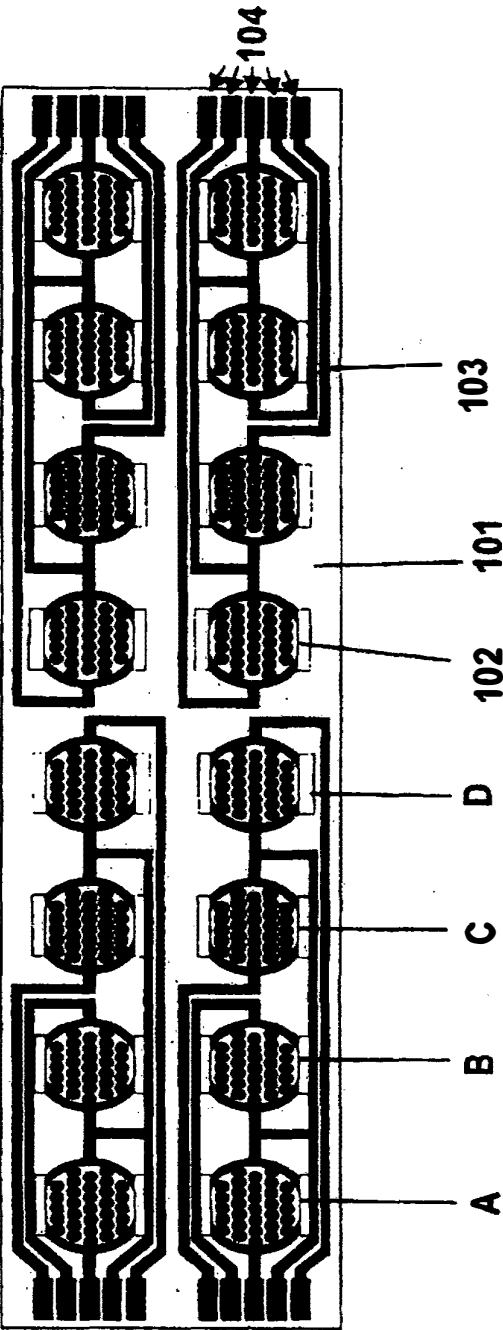


FIG. 1A

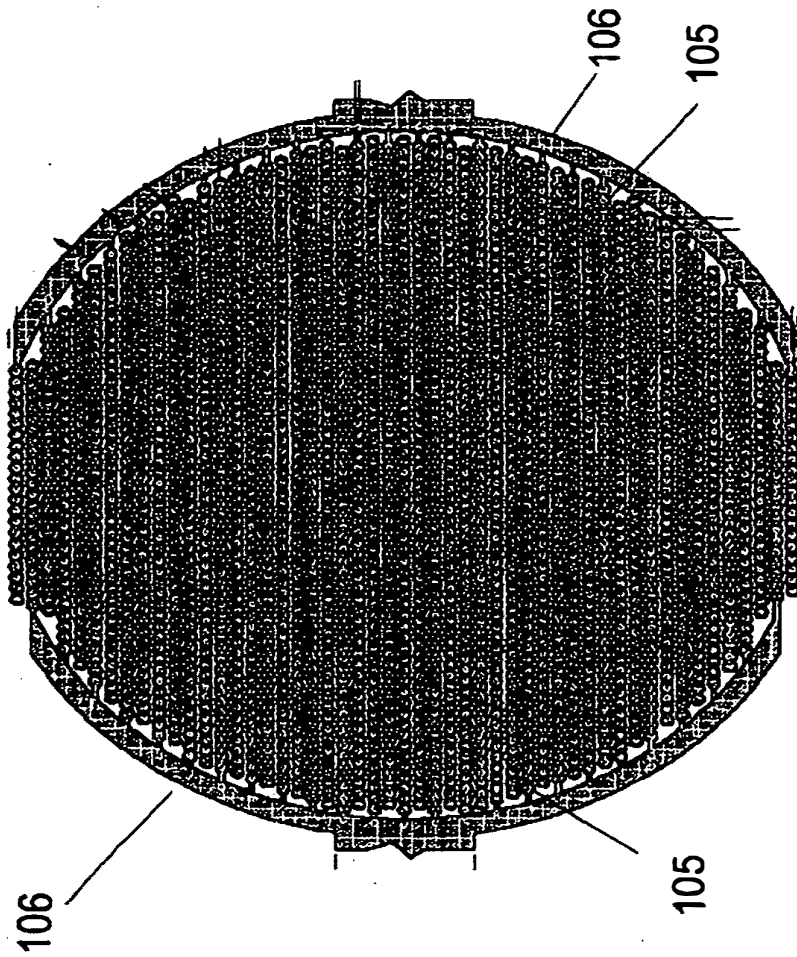


FIG. 1B

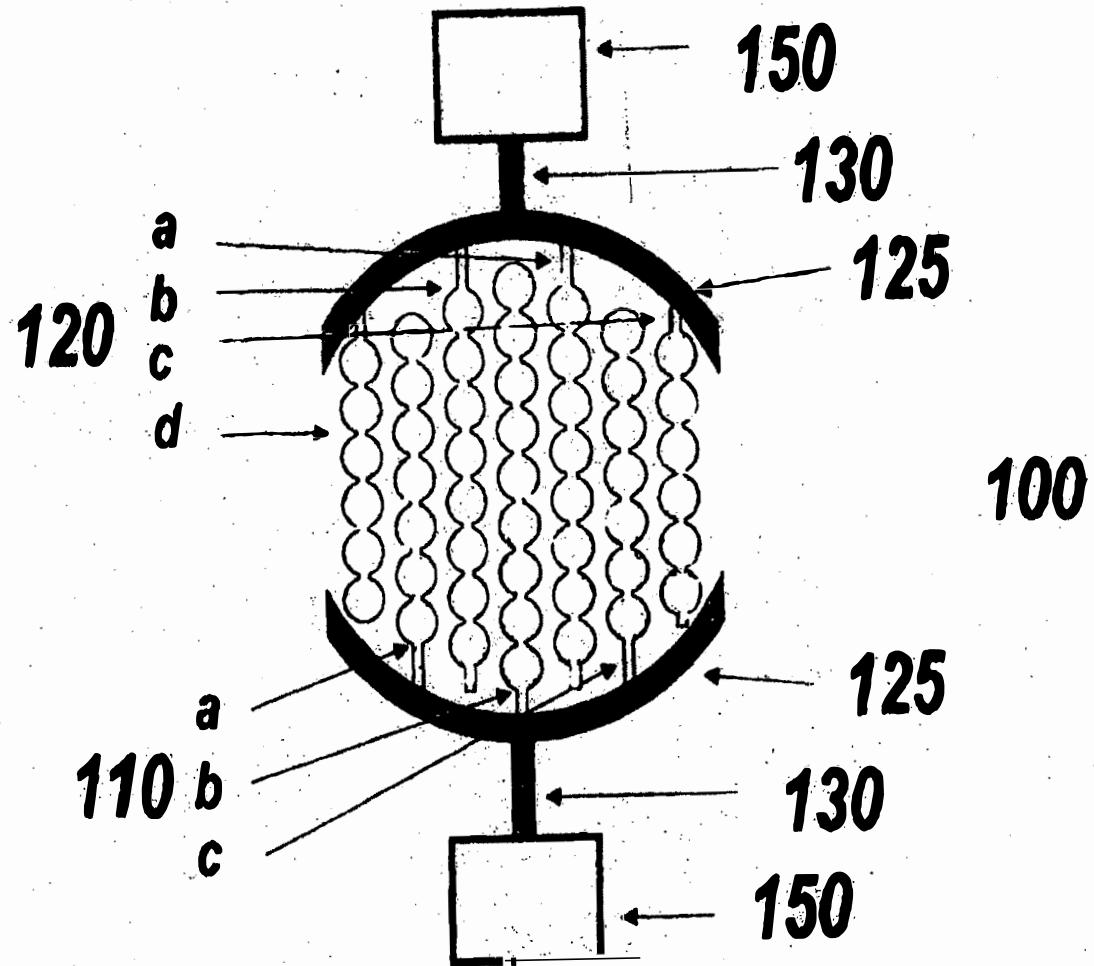


FIG. 2A

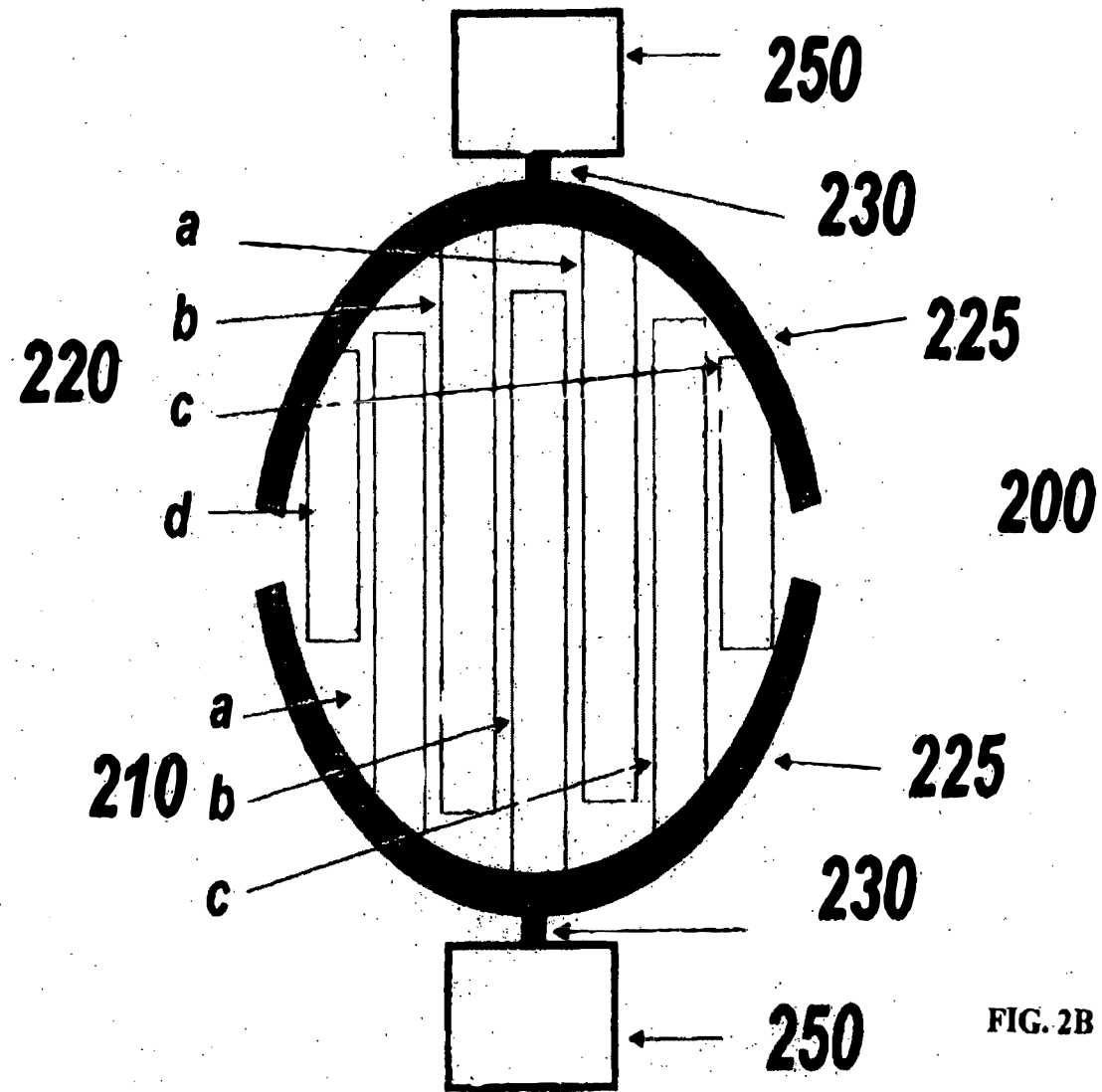


FIG. 2B

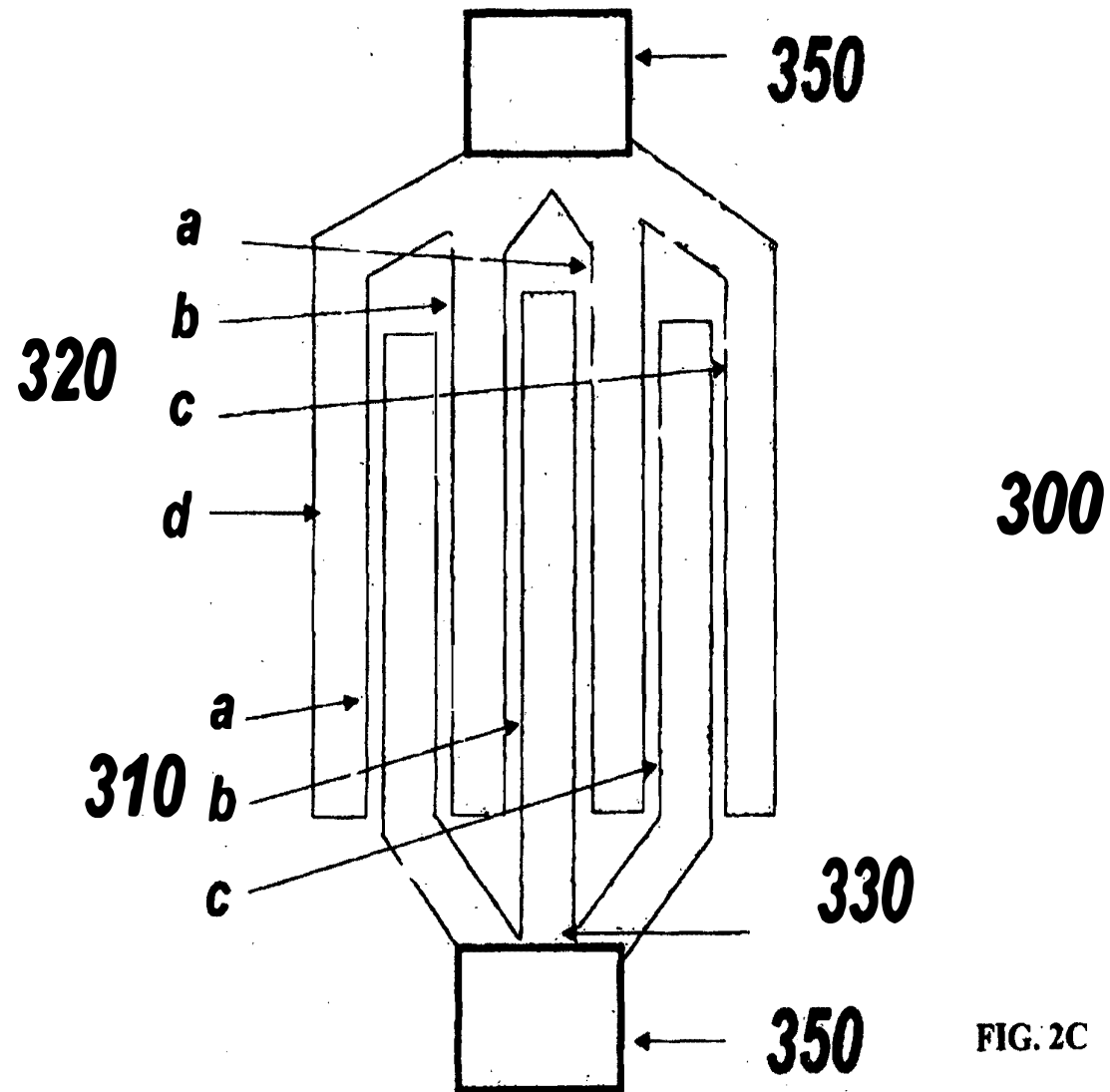


FIG. 2C

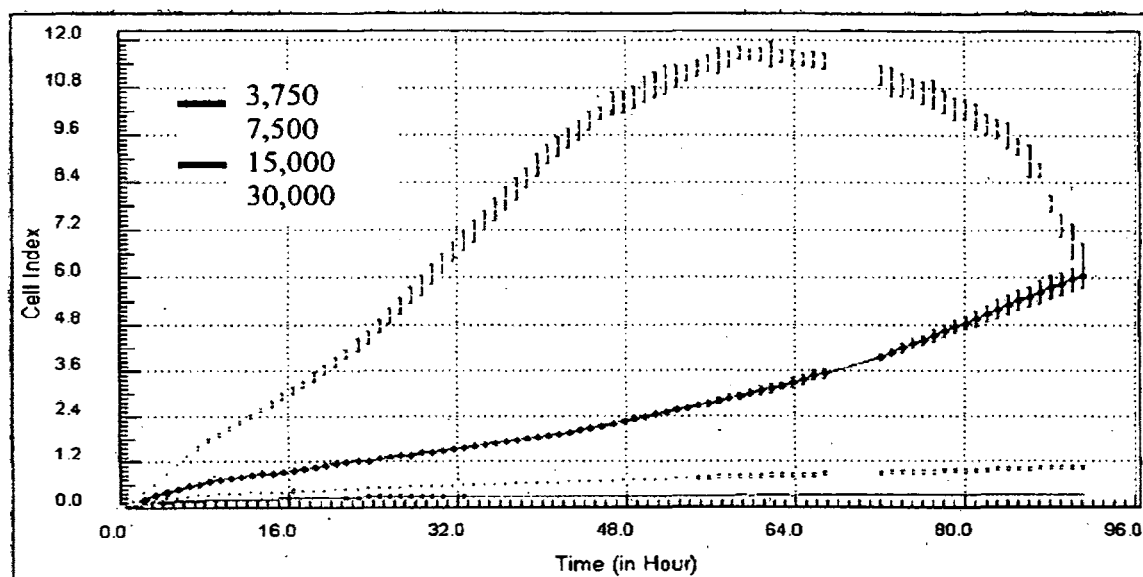
FIG. 3**Titration of ES-derived cardiomyocytes using the ACEA RT-CES system**

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

FIG. 4A

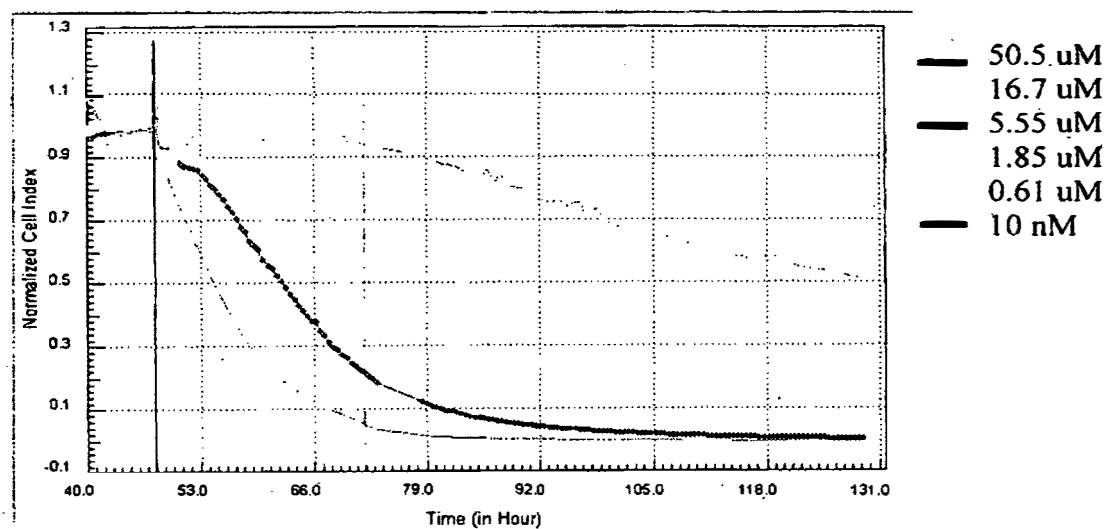


FIG. 4B

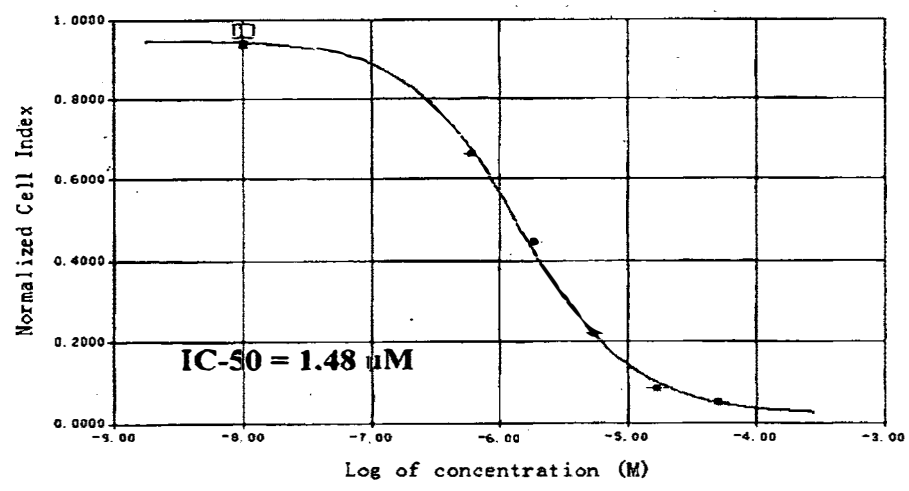


FIG. 5. Detection of morphological changes associated with ES-derived cardiomyocytes induced by isoproterenol, a β_2 adrenergic receptor agonist, using the RT-CES system

Fig. 5A.

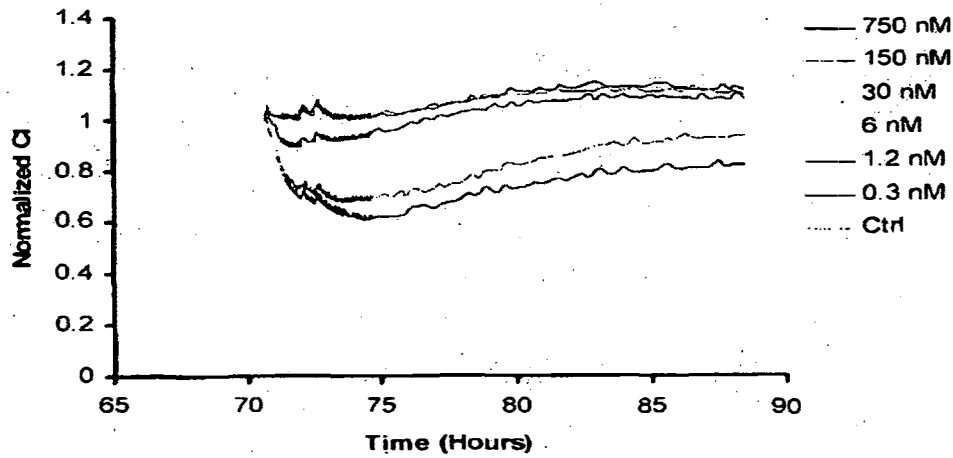


Fig. 5B.

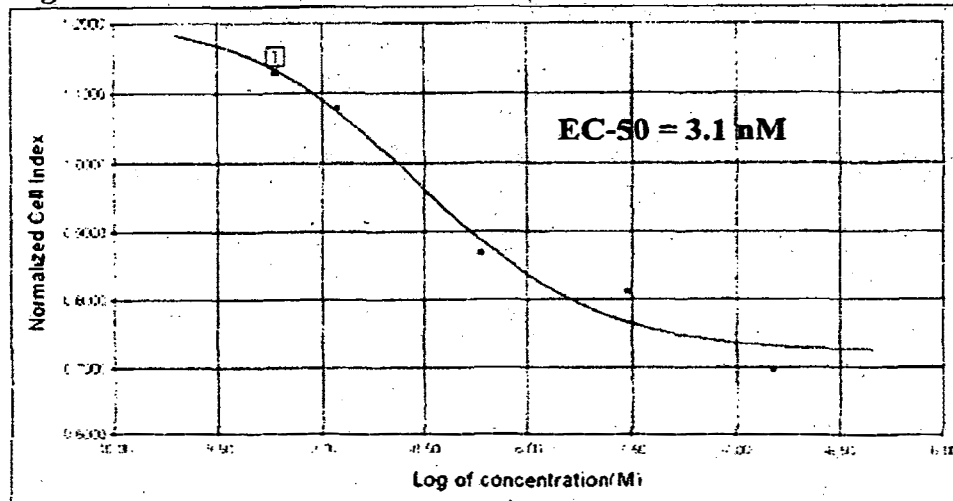


FIG. 6

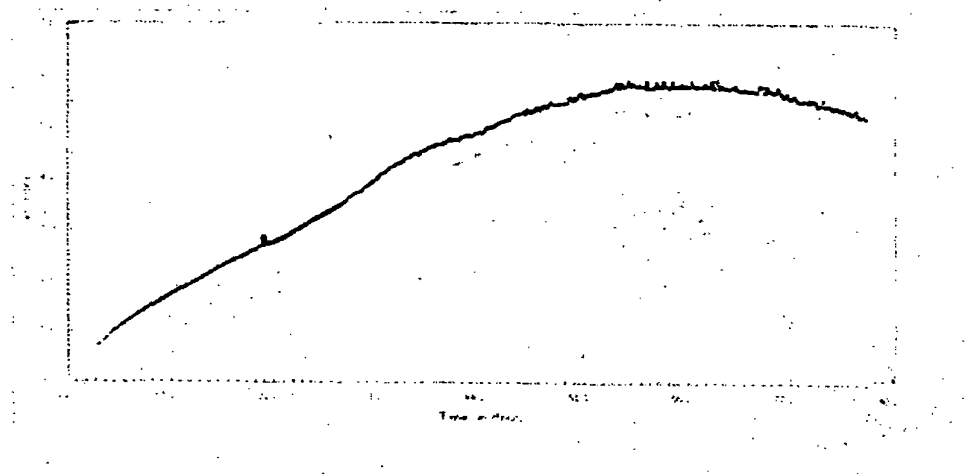


FIG. 7

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.

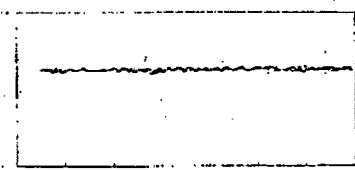
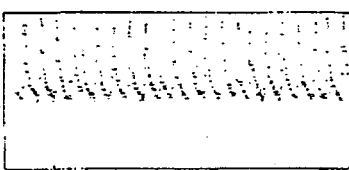
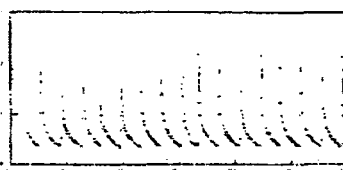
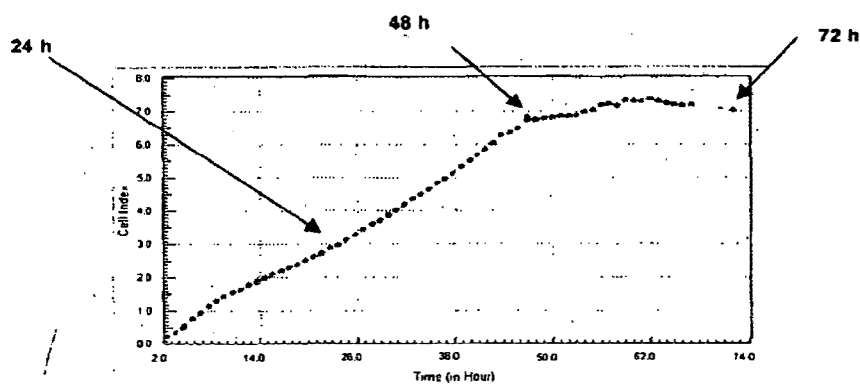
7A**7B****7C****7D**

FIG. 8A

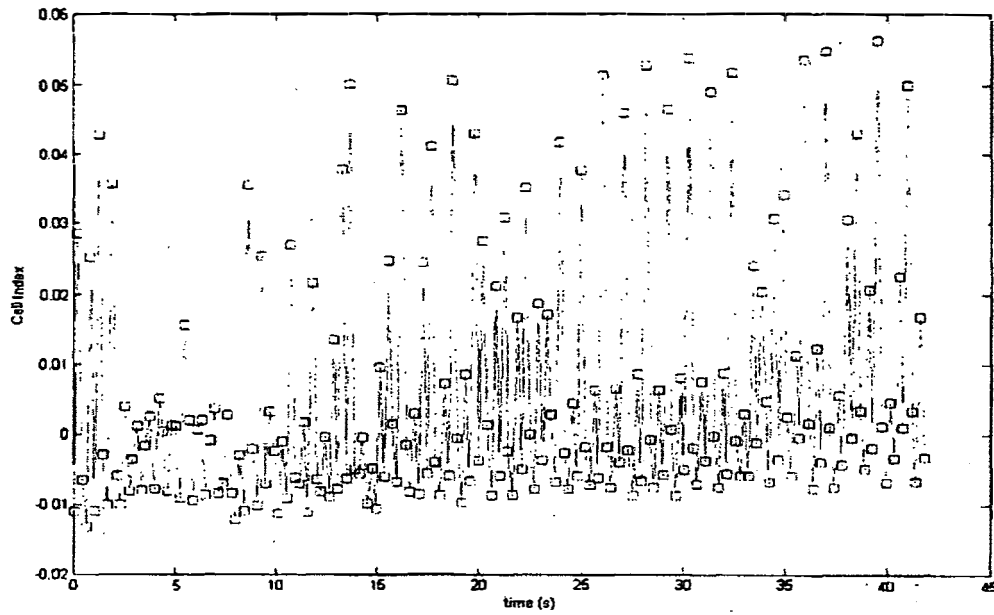


FIG. 8B

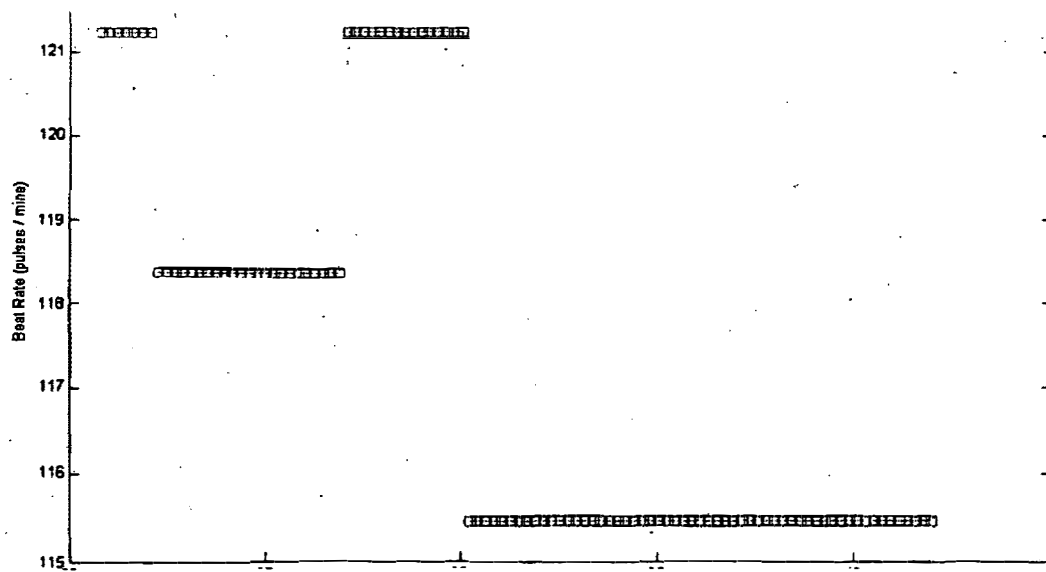


FIG. 9 Detection of cardiomyocyte beating using the ACEA RT-CES system for the mouse cardiomyocytes treated with 4.4 μM sotalol. In Figures 9C and 9D, the time resolution between two adjacent points is 40 milliseconds. In other words, a second in Figures 9C and 9D is equivalent to 40-millisecond.

FIG. 9A

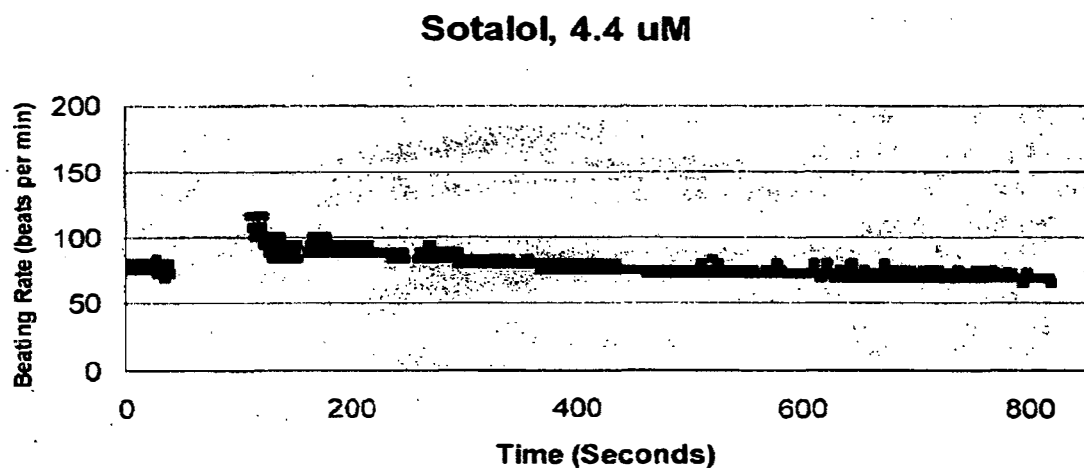


FIG. 9B

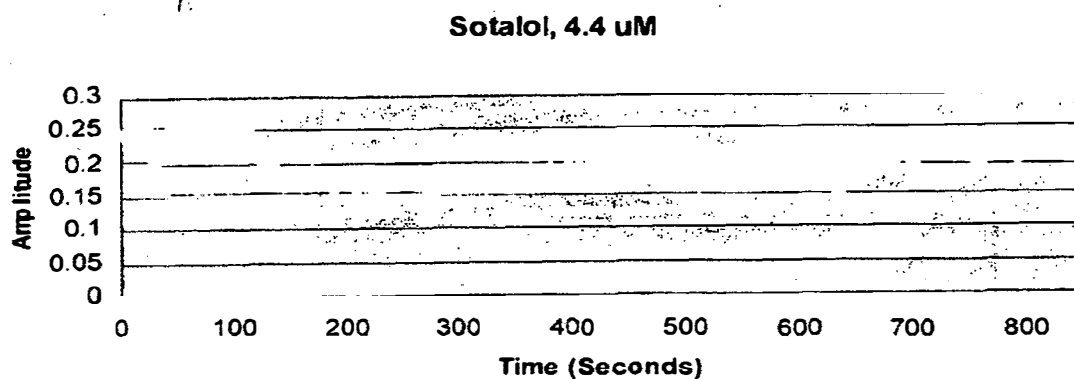


FIG. 9C

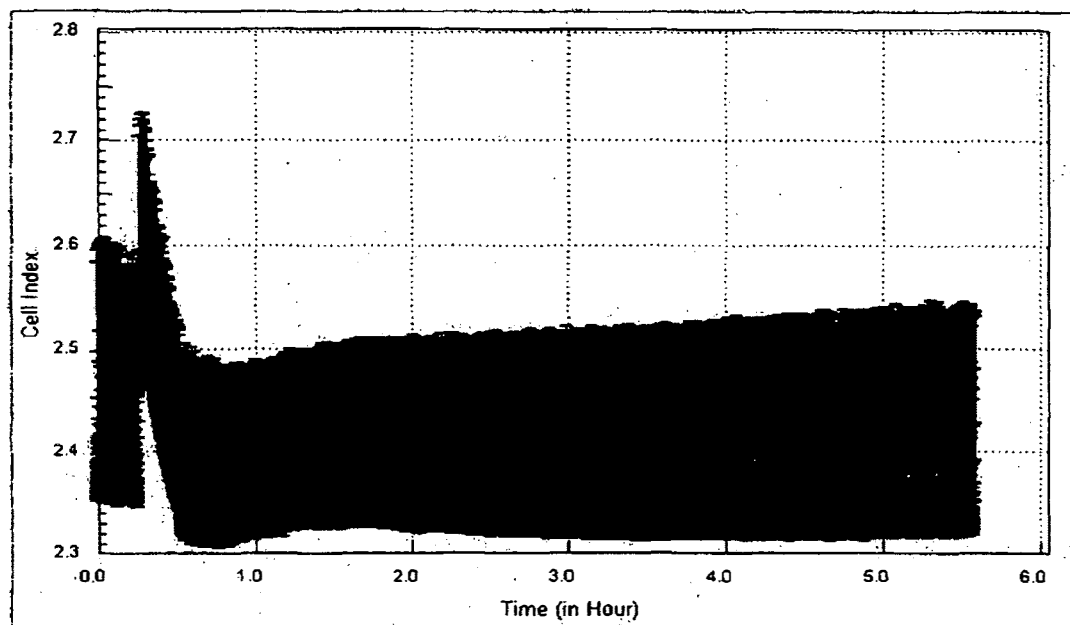


FIG. 9D

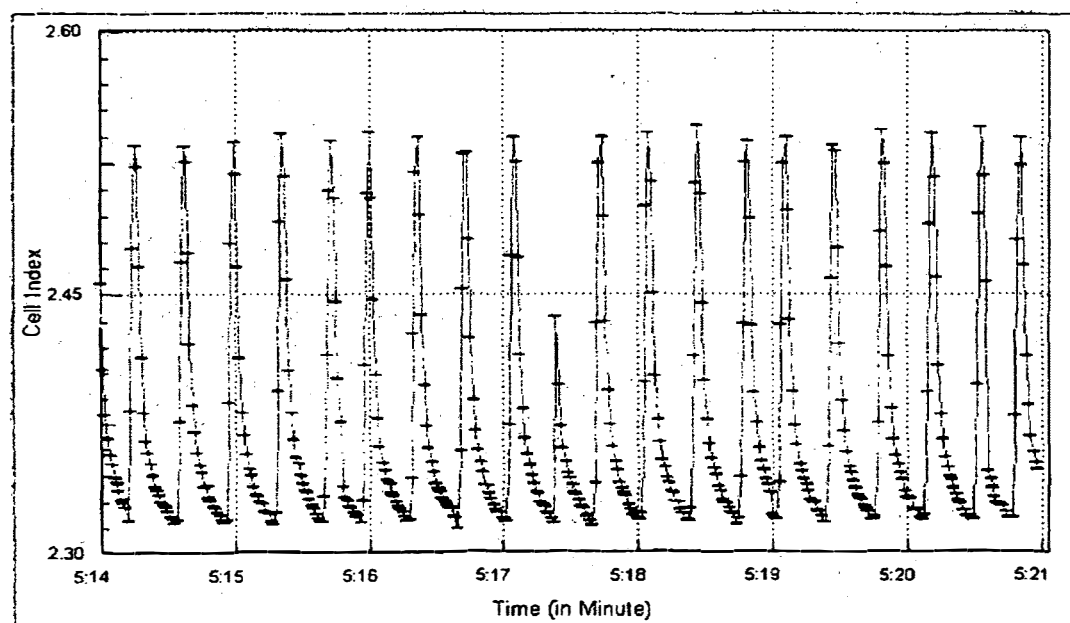


FIG. 10A

Assessment of agents which slow down heart rate and cardiomyocyte beating

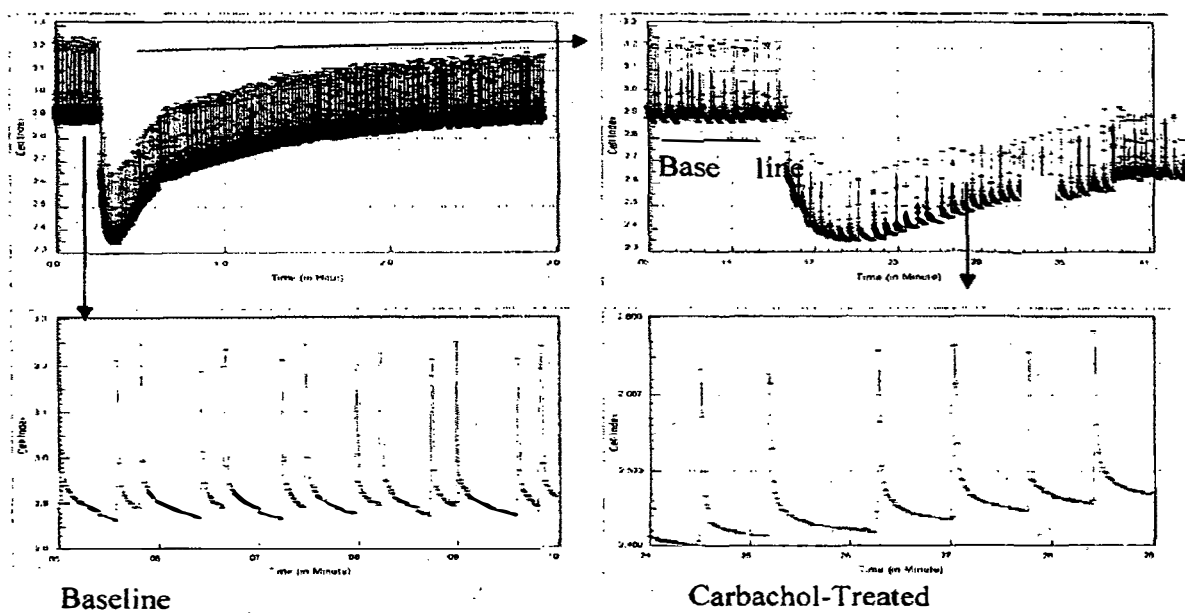


FIG. 10B

Analysis of drugs which increase heart rate and cardiomyocyte beating

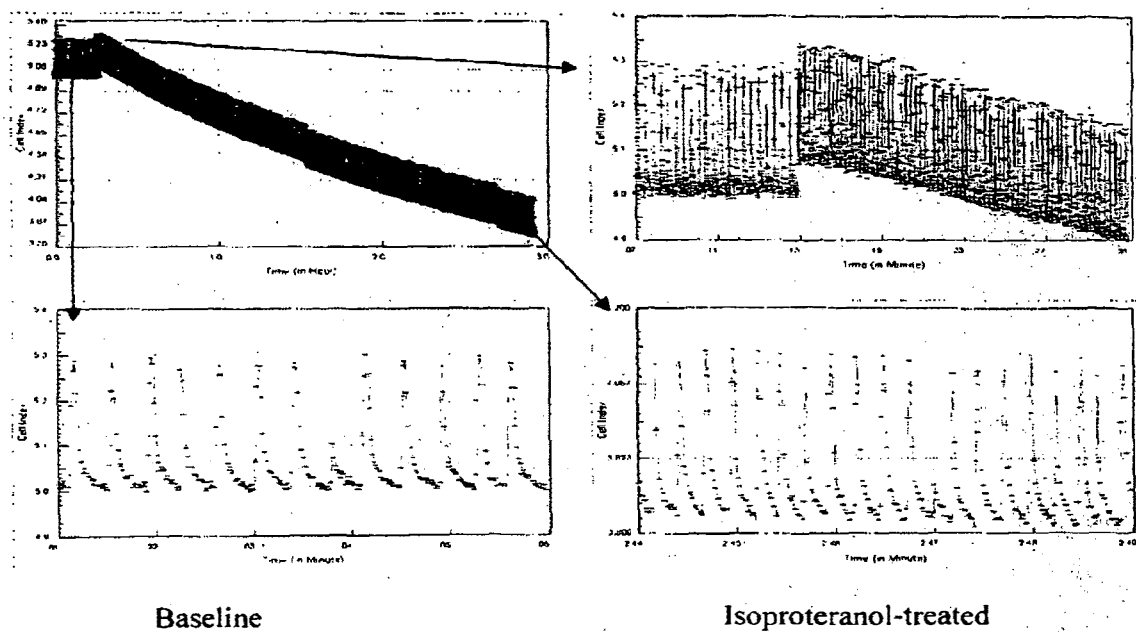


FIG. 11A

Sotalolol 33.3 μ M (0.033% DMSO)

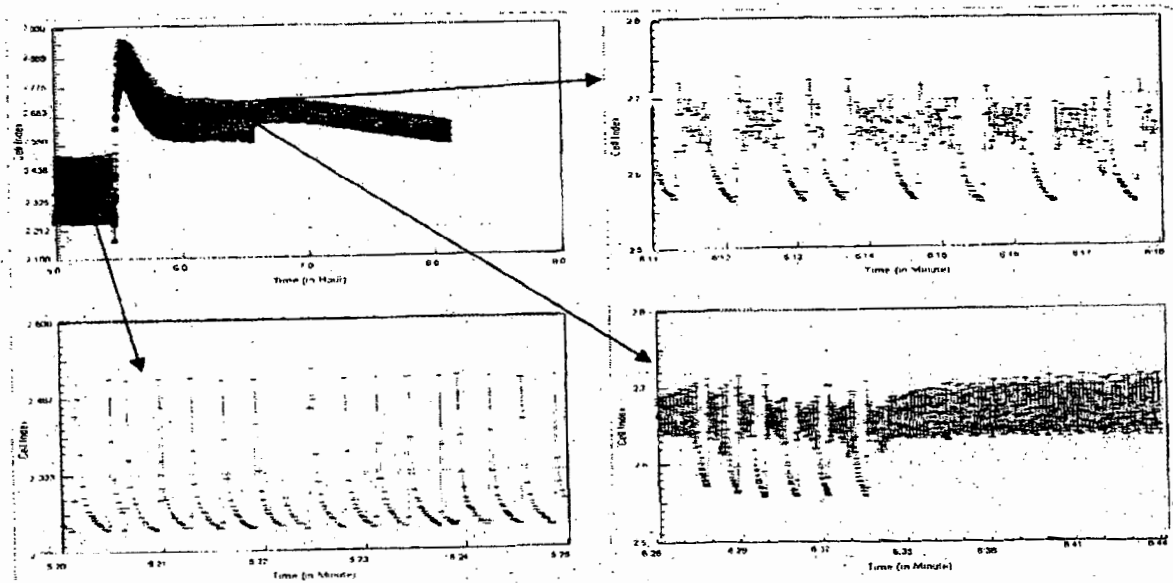


FIG. 11B

Astemizole 400 nM (0.004% DMSO)

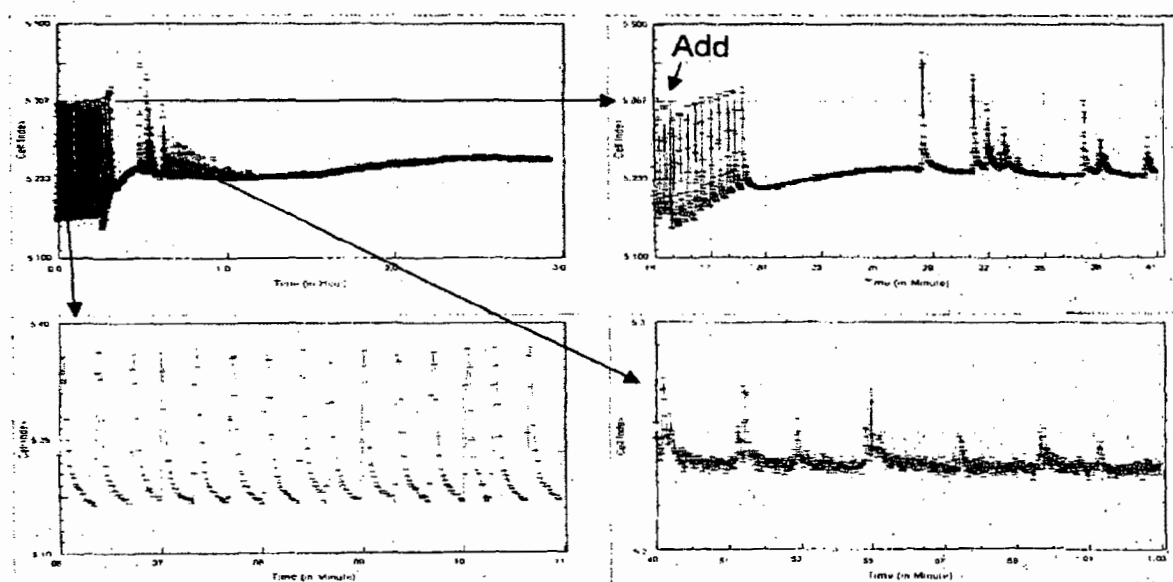


FIG. 11C

Terfenadine 200 nM (0.065%DMSO)

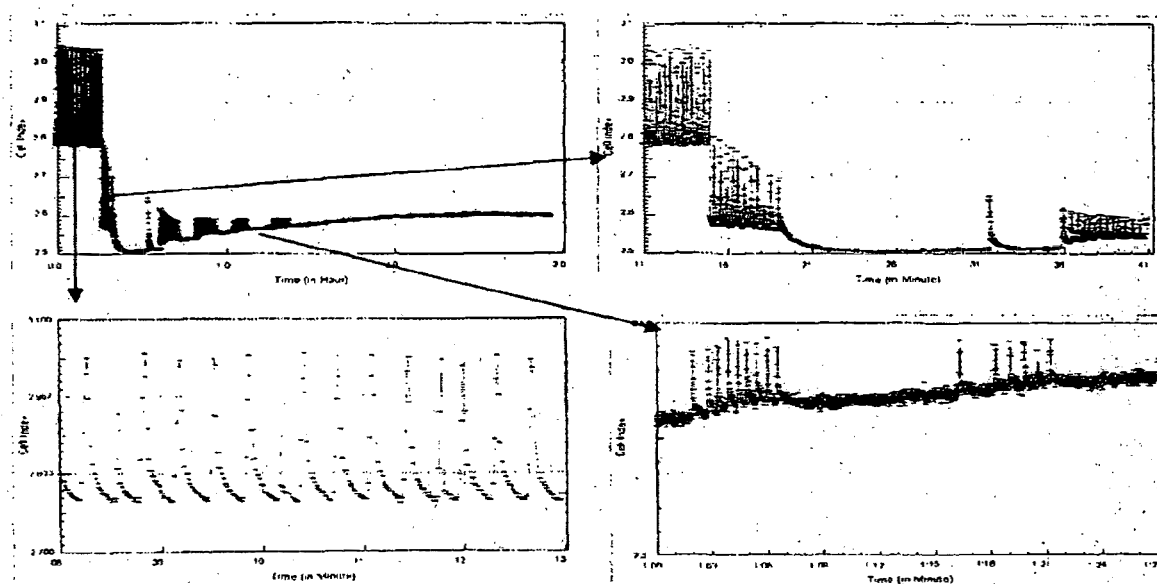


FIG. 11D

Erythromycin 13.3 μ M (0.13% DMSO)

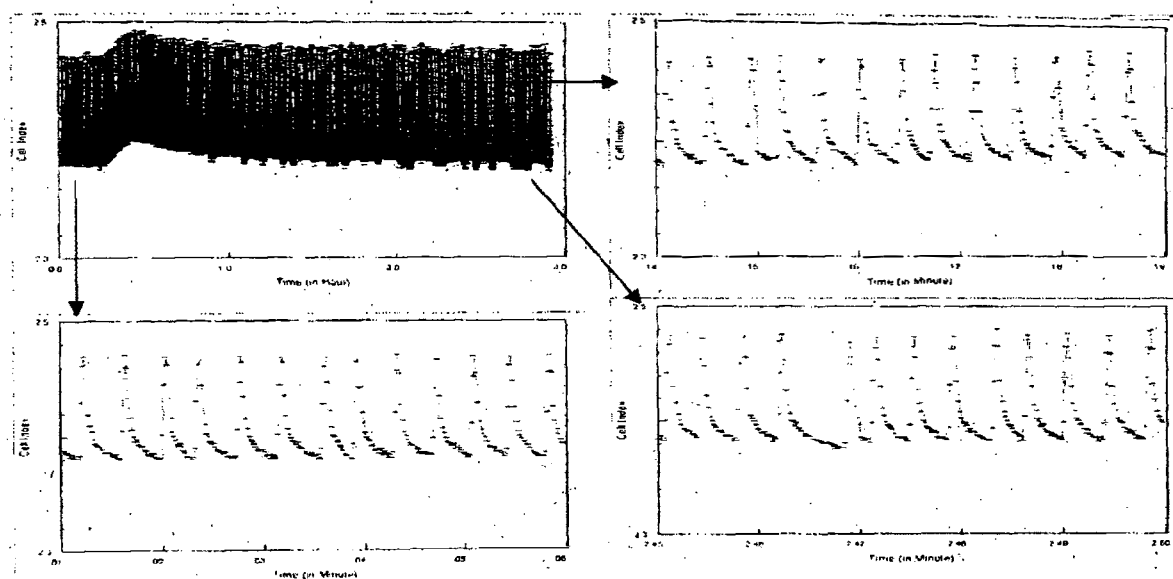


FIG. 11E

Moxifloxacin 20 μ M (0.3% DMSO)

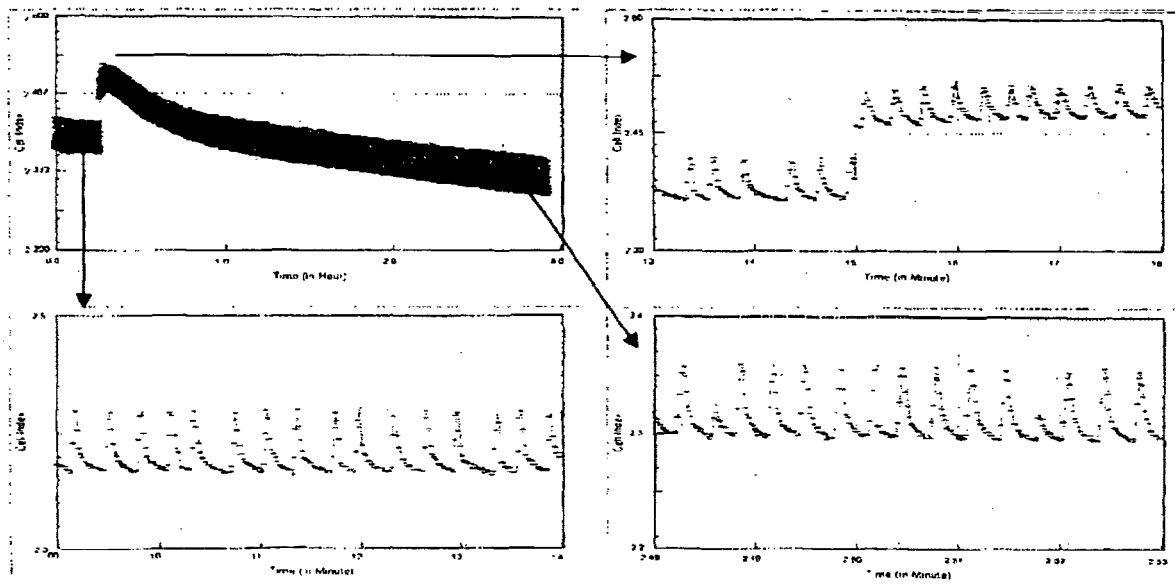


FIG. 11F

Pentamidine 20uM (0.2%DMSO)

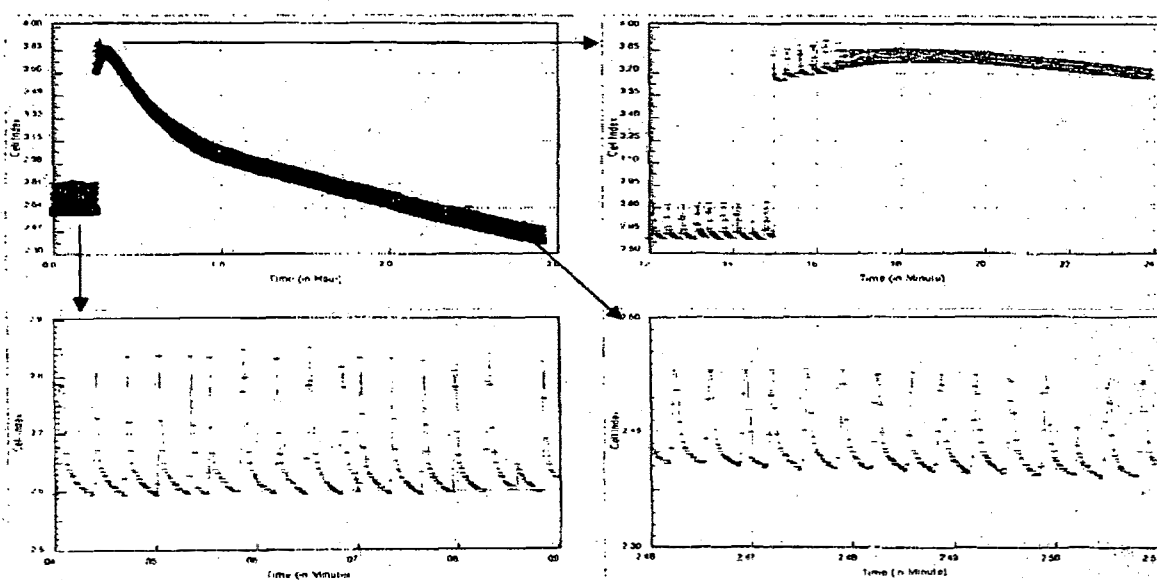


FIG. 11G

Amitriptyline 4.4uM(0.04 % DMSO)

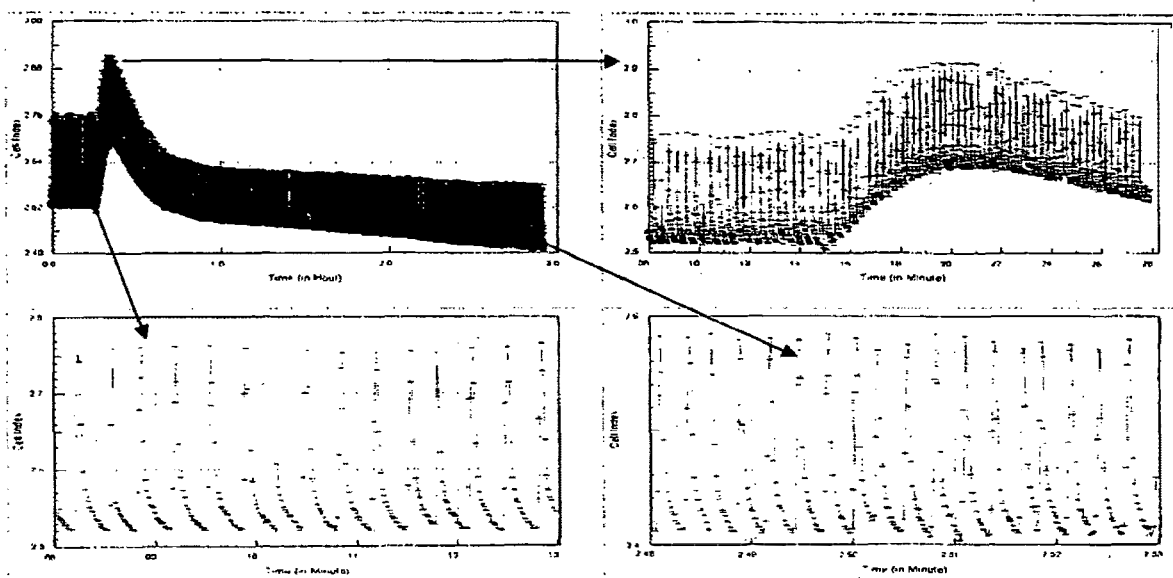


FIG. 11H

Verapamil 130 nM (0.0013% DMSO)

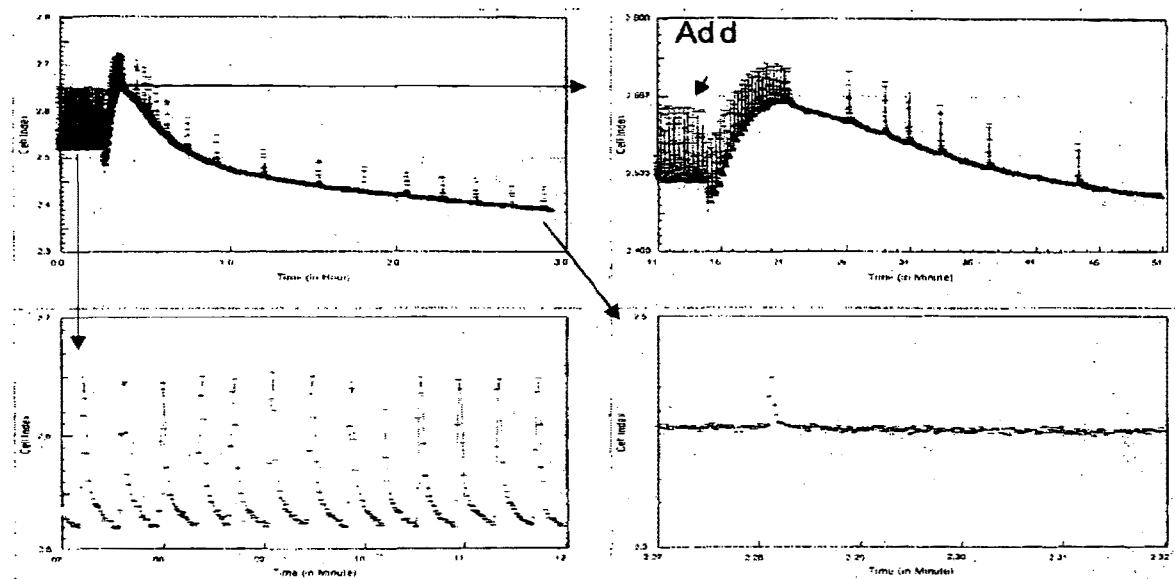


FIG. 11I

Rosiglitazone 13.3 uM (0.13% DMSO)

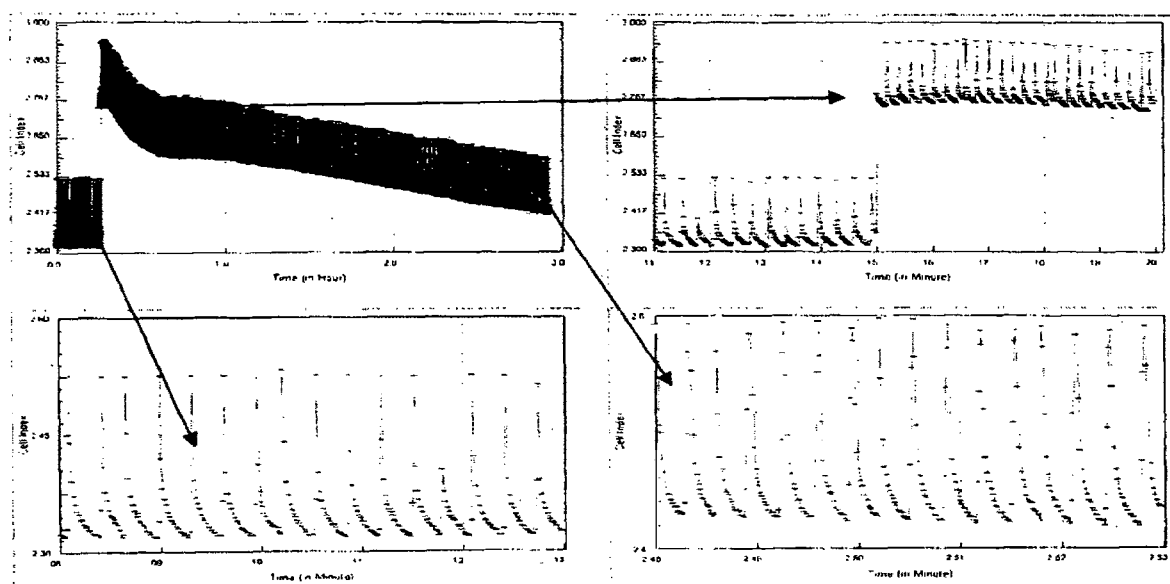


FIG. 11J

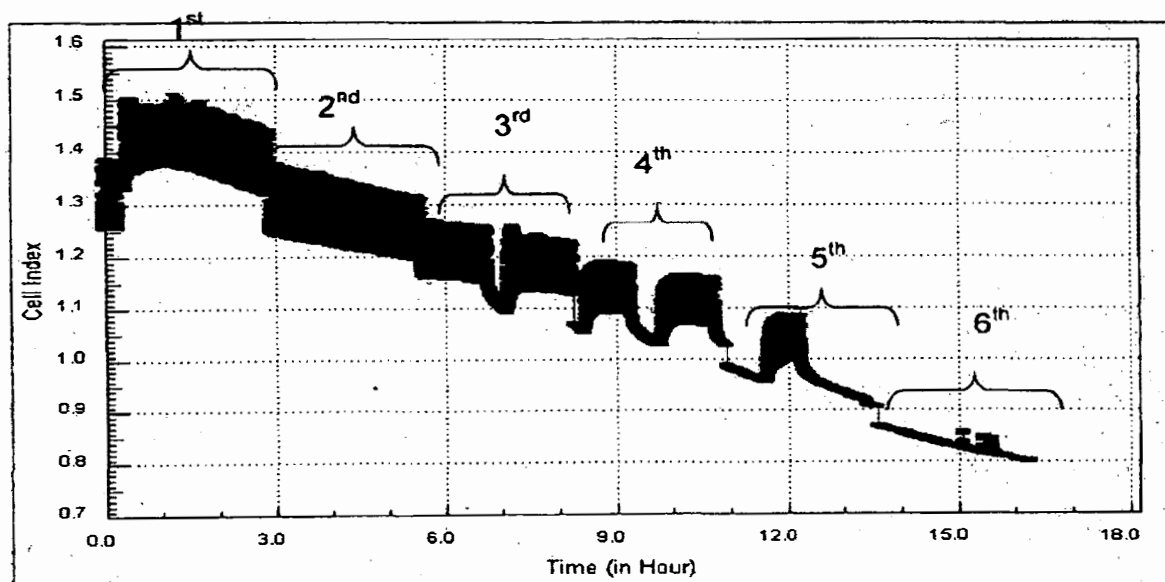
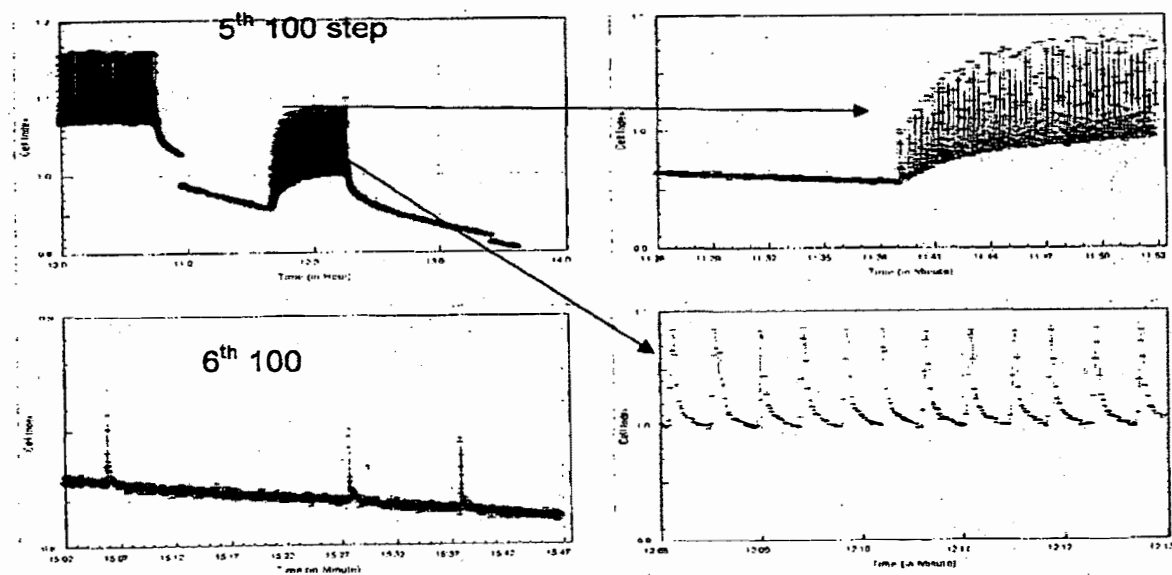
Rofecoxib 13.3 μ M

FIG. 11K

Celecoxib 4.4 μ M

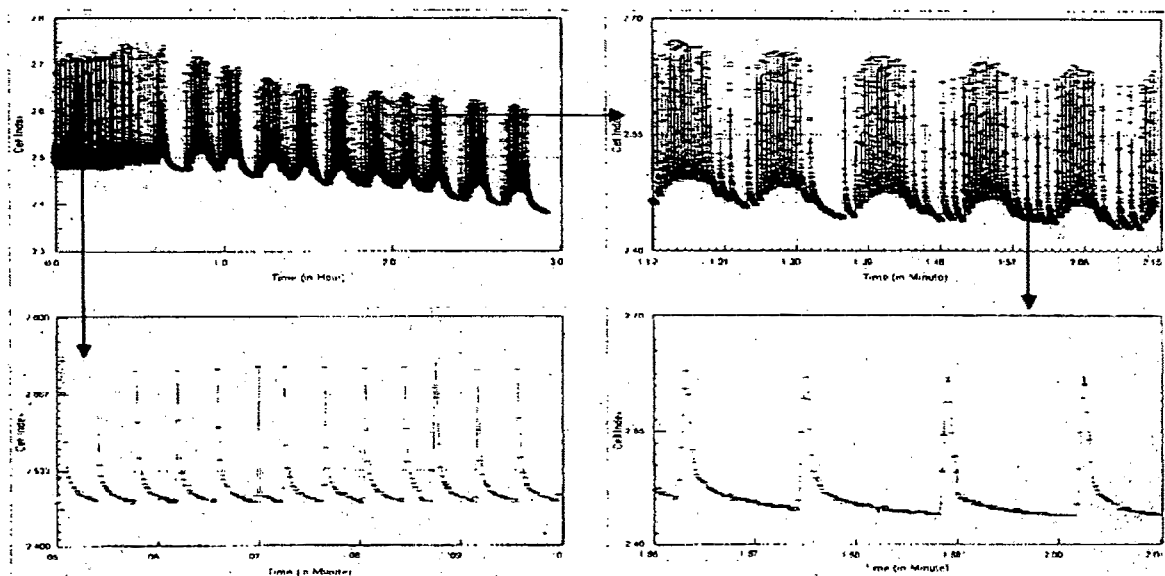


FIG. 11L

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

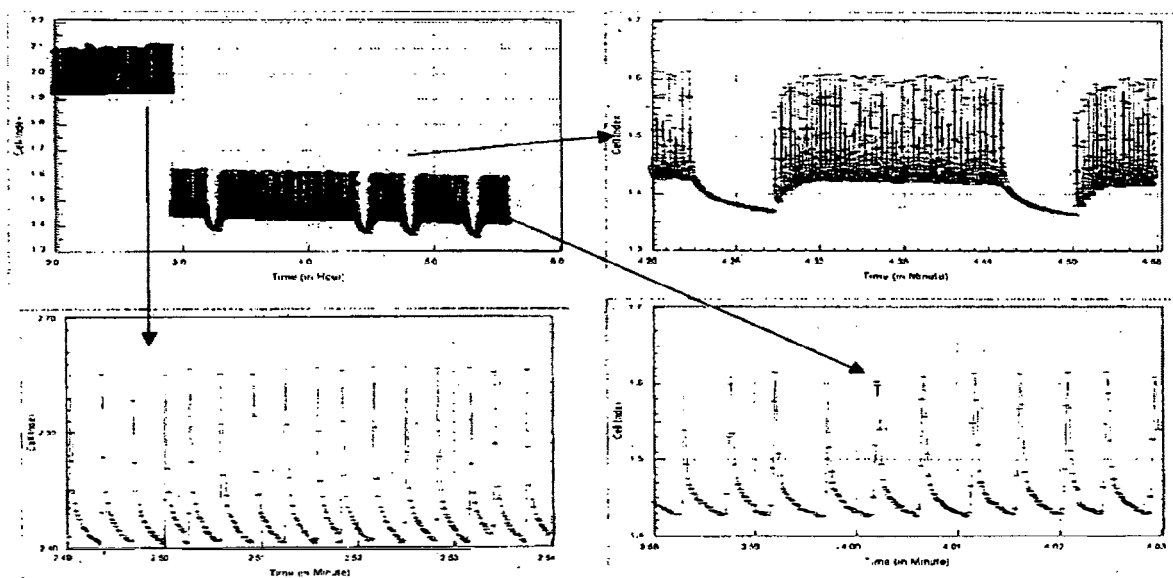


FIG. 11M

Cyclosporine A 13.3uM (0.13% DMSO)

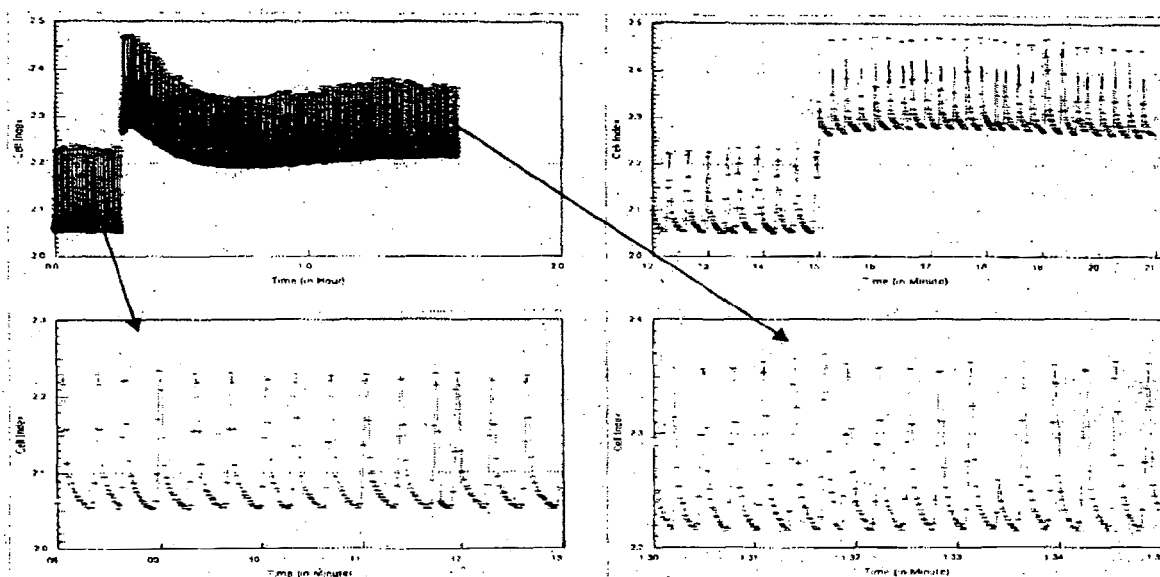


FIG. 11N

Propranolol 4.4uM (0.04% DMSO)

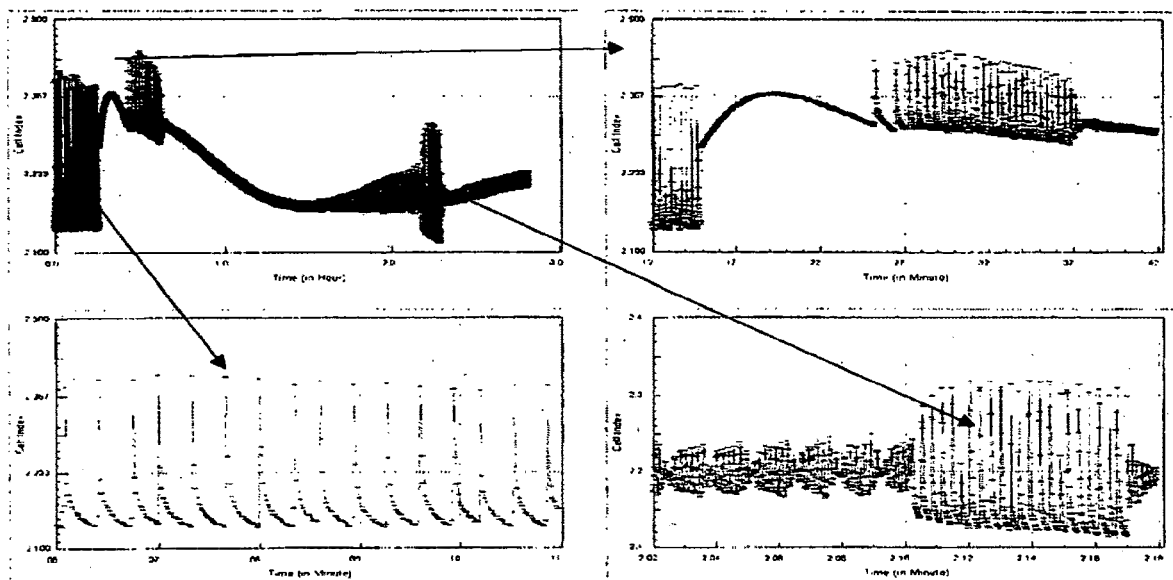


FIG. 110

E4031 9.1 nM

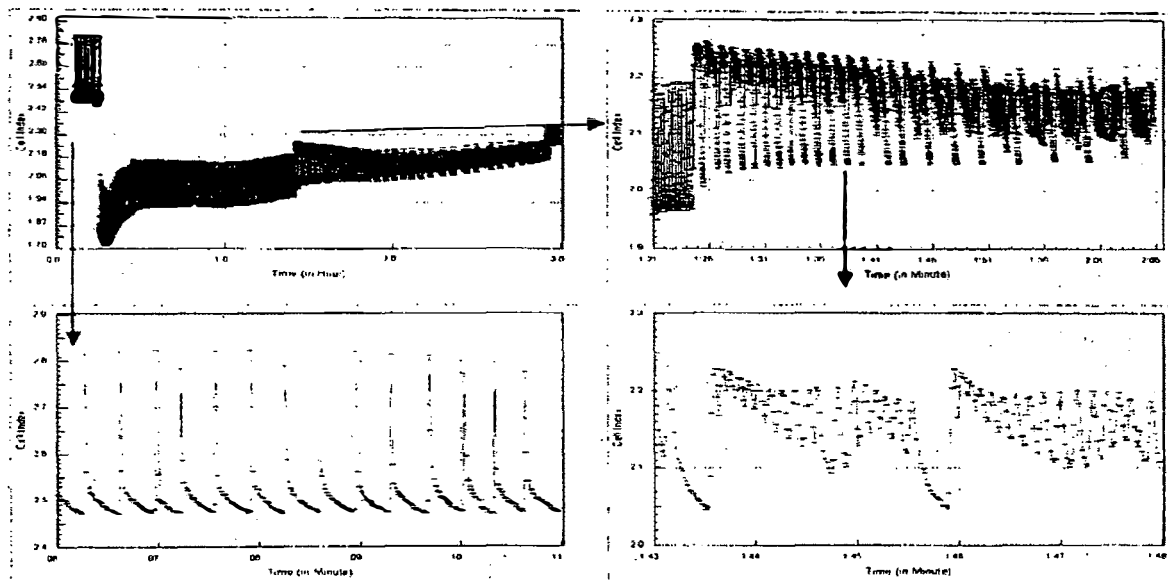


FIG. 11P

DDT 8 μ M (0.08% DMSO)

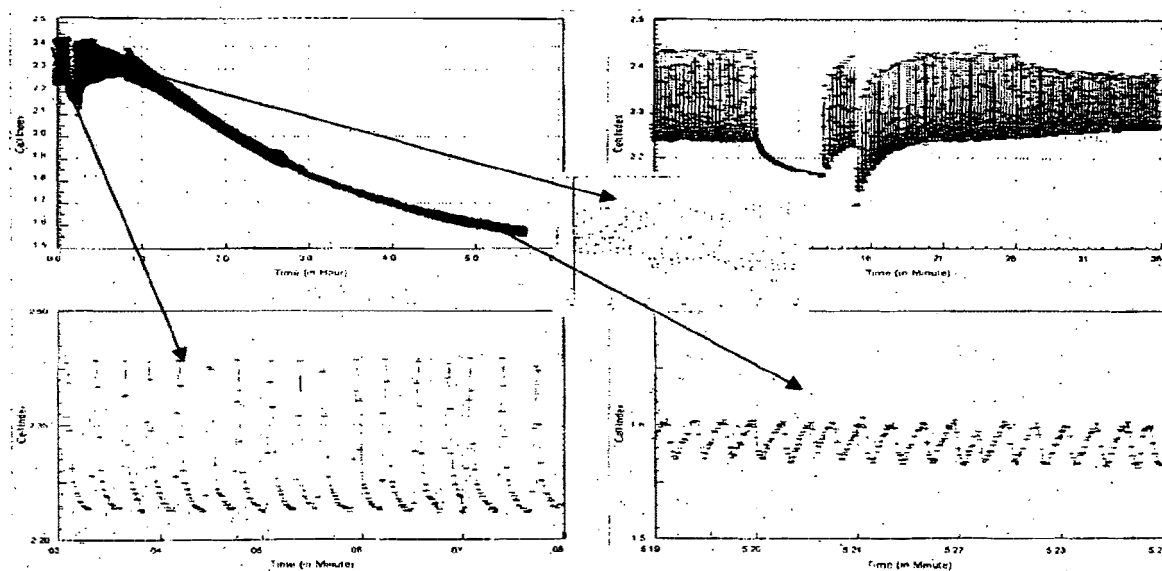


FIG. 11Q

PCB 8uM (DMSO 0.08%)

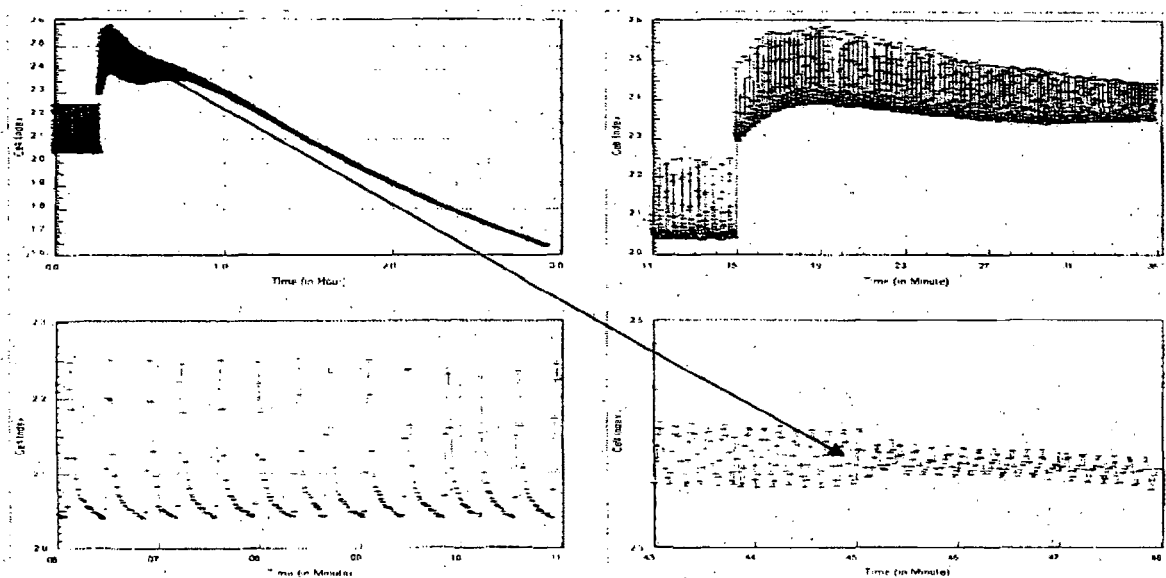


FIG. 11R

Endosulfan 8uM (0.08% DMSO)

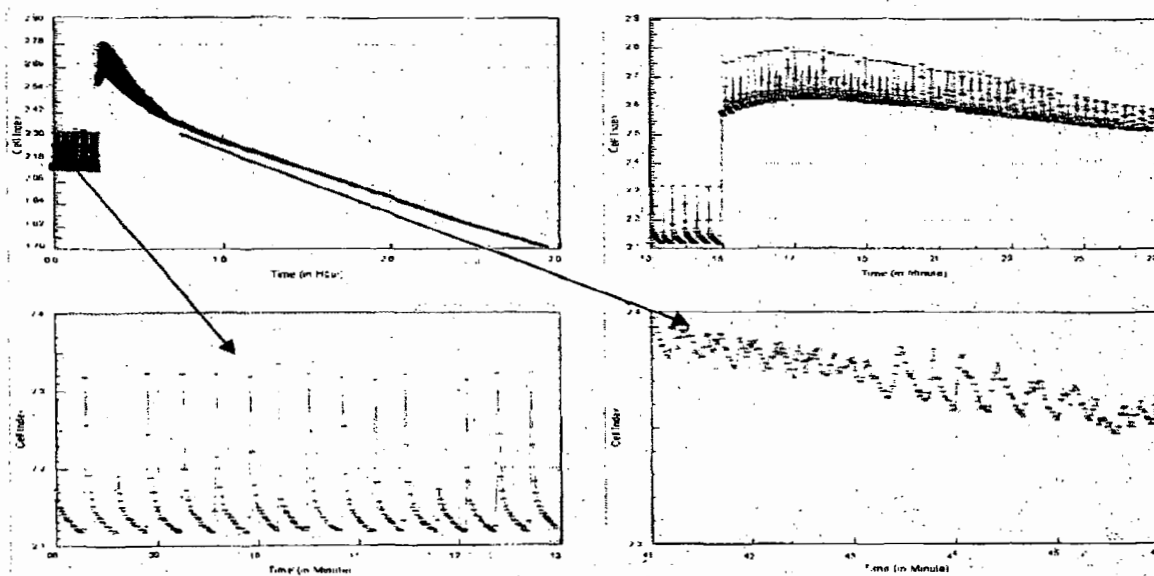


FIG. 11S

DMSO 0.13%

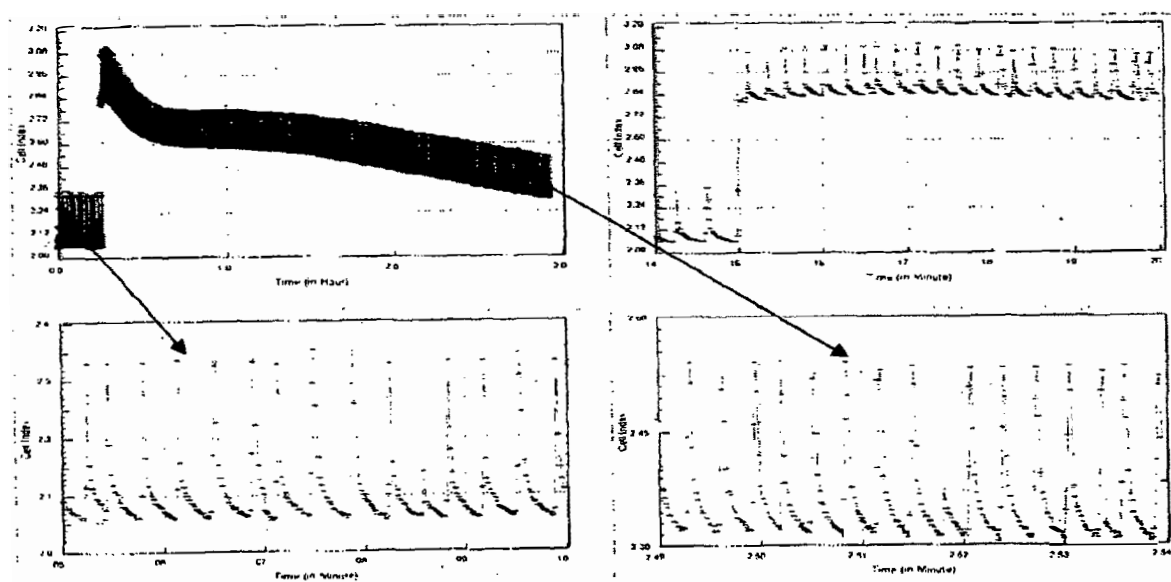


FIG. 12

Cardiotoxic Patterns

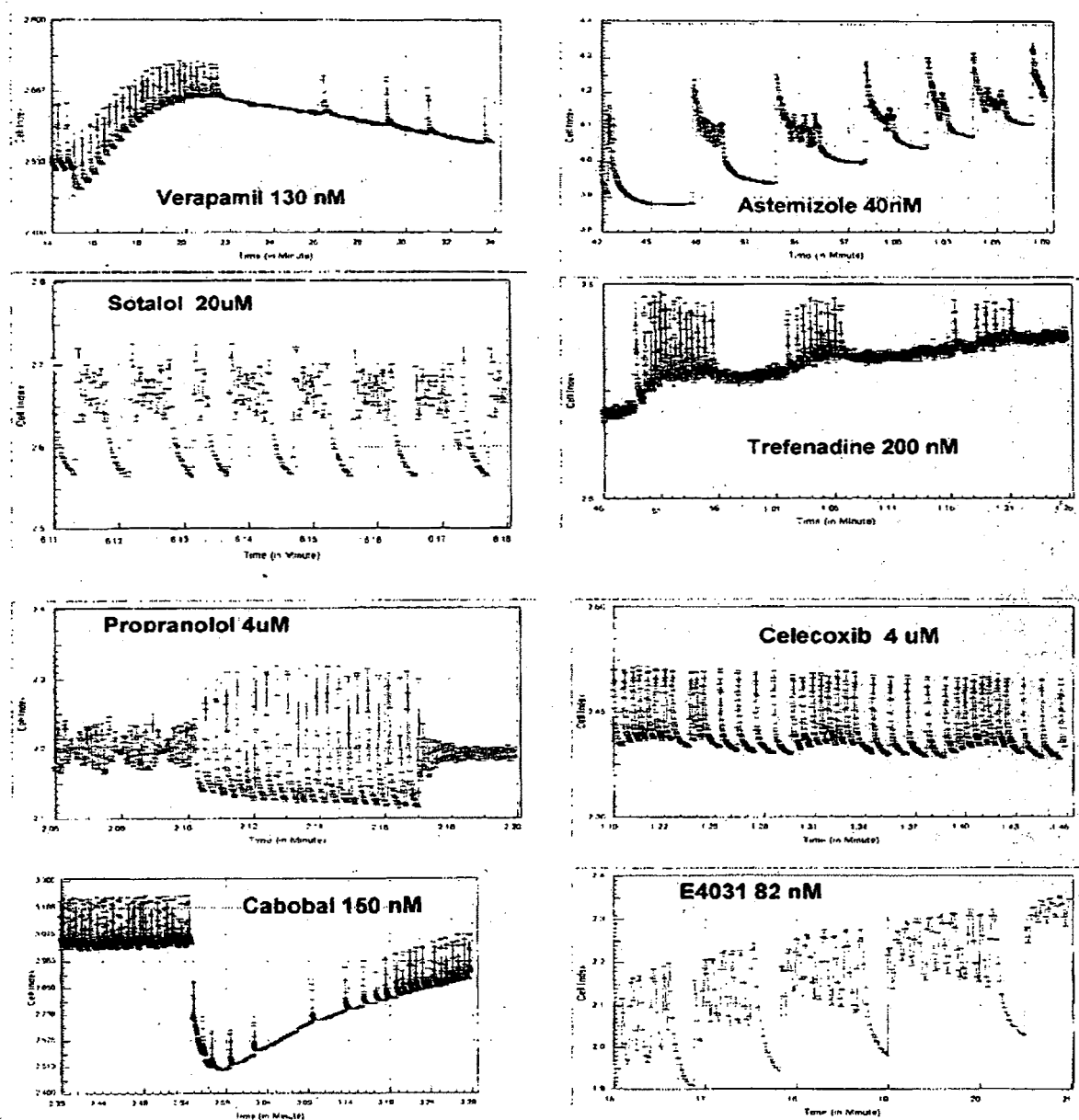


FIG. 13.

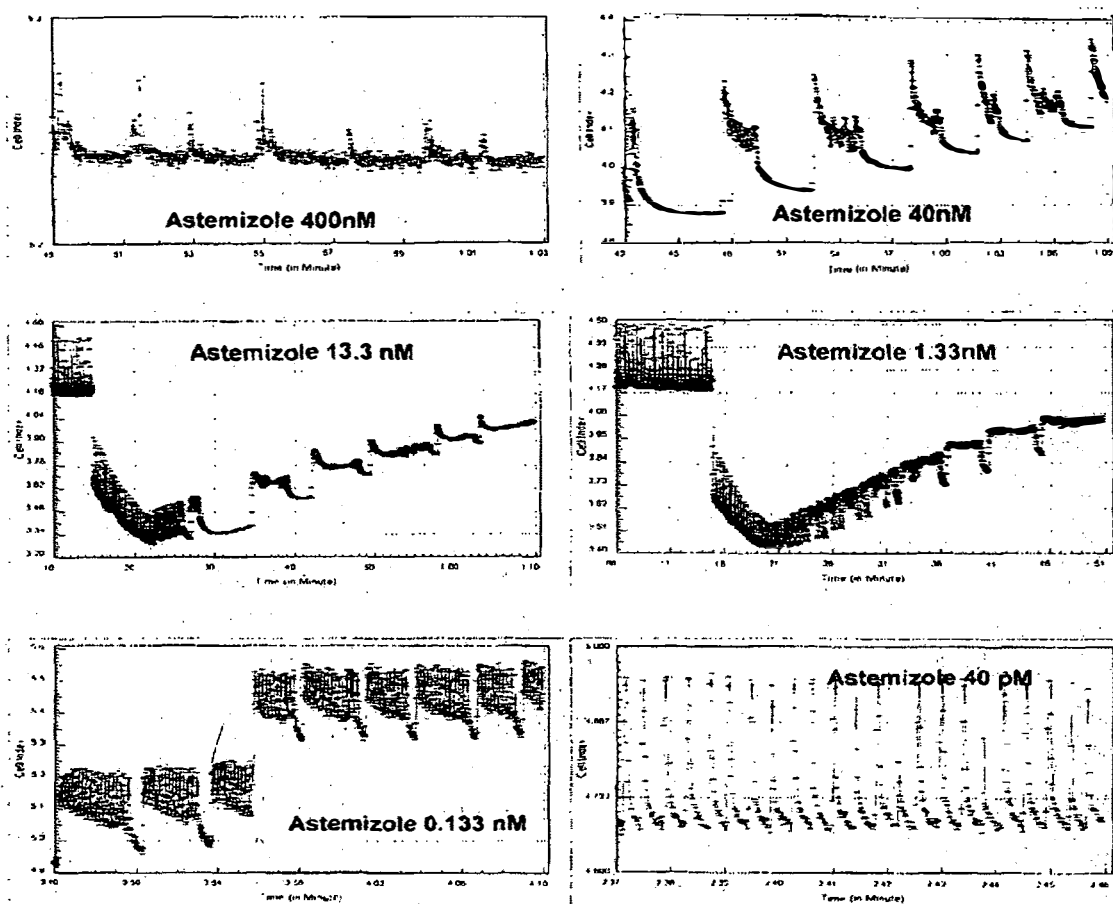


FIG. 14

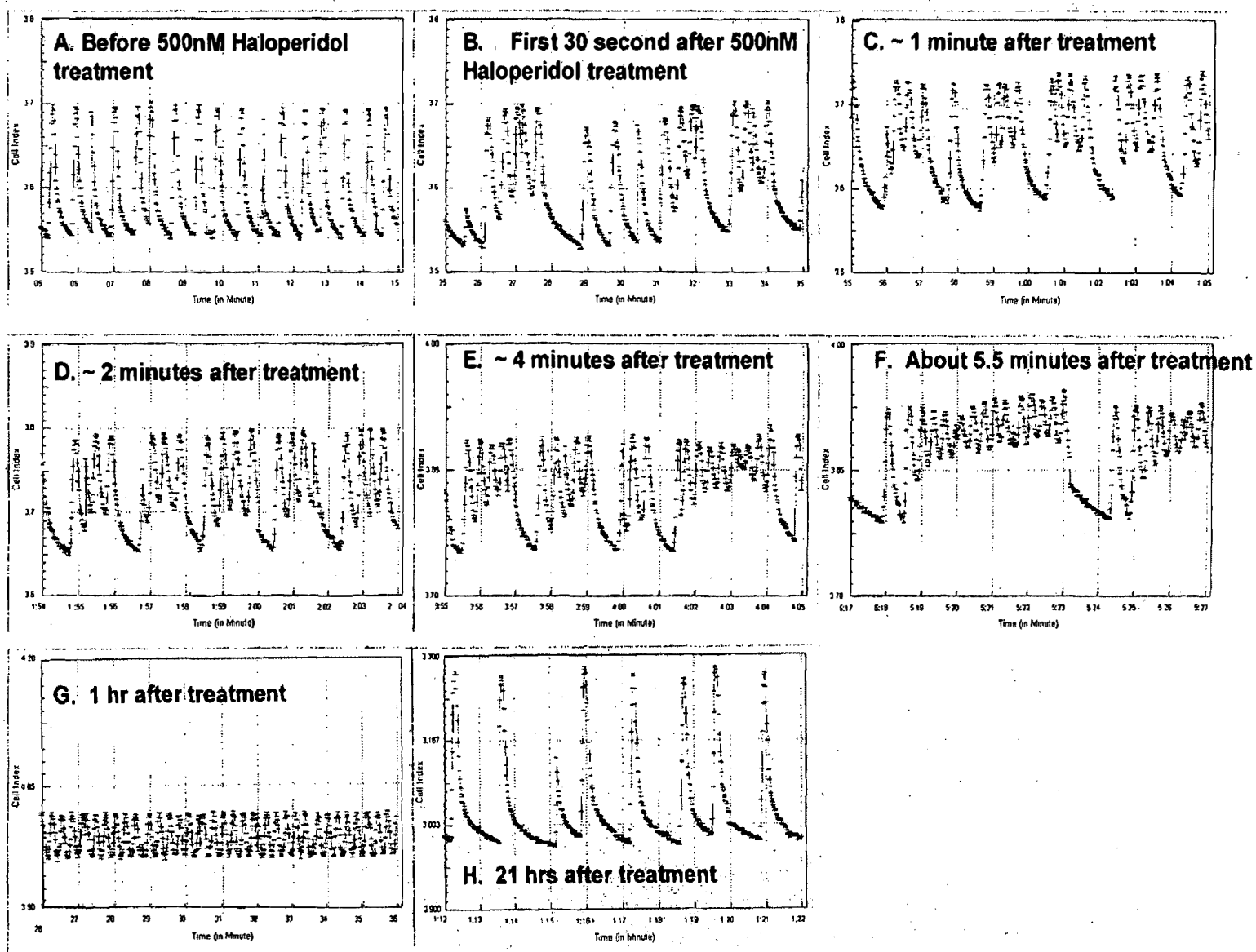


FIG. 15

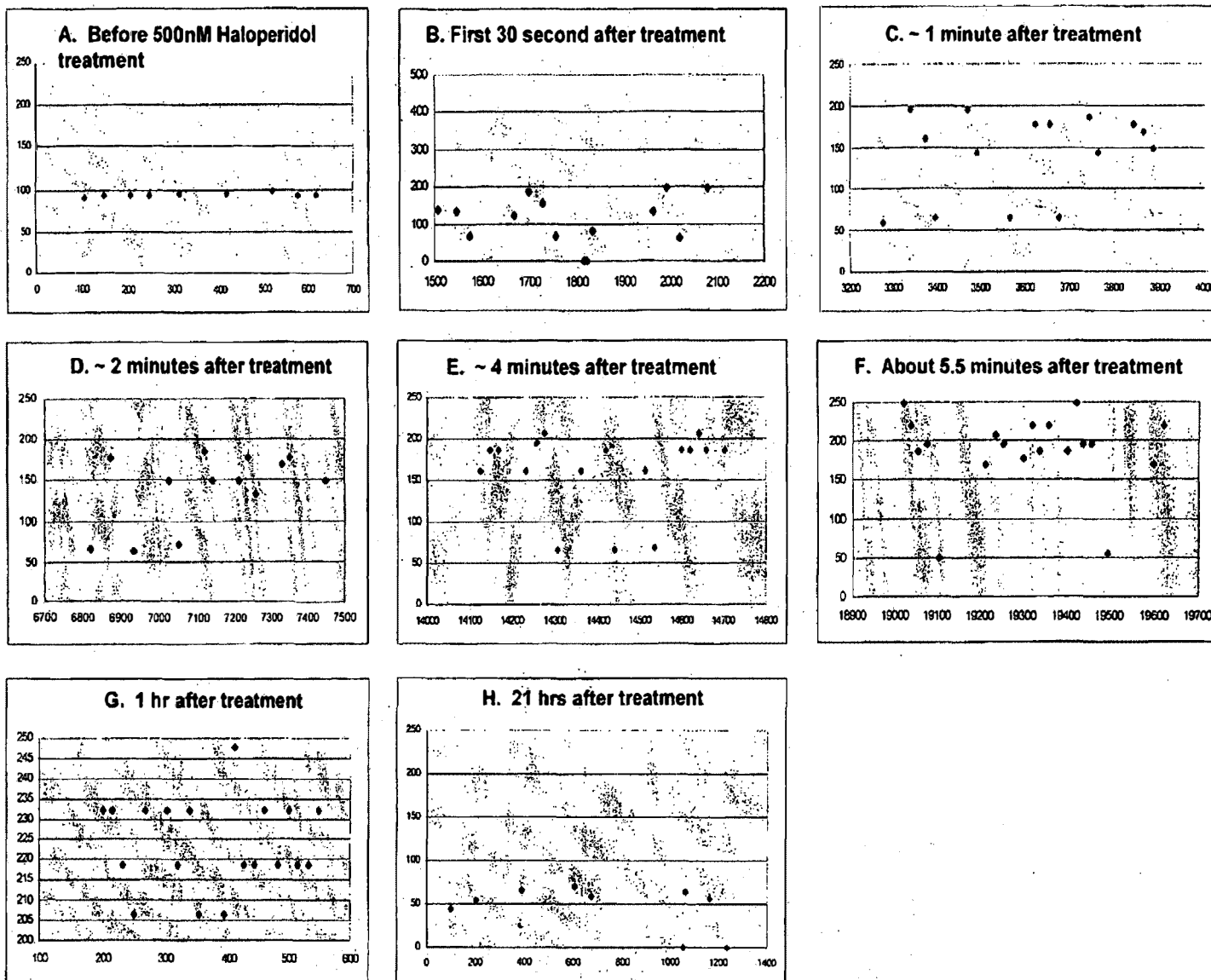


FIG. 16

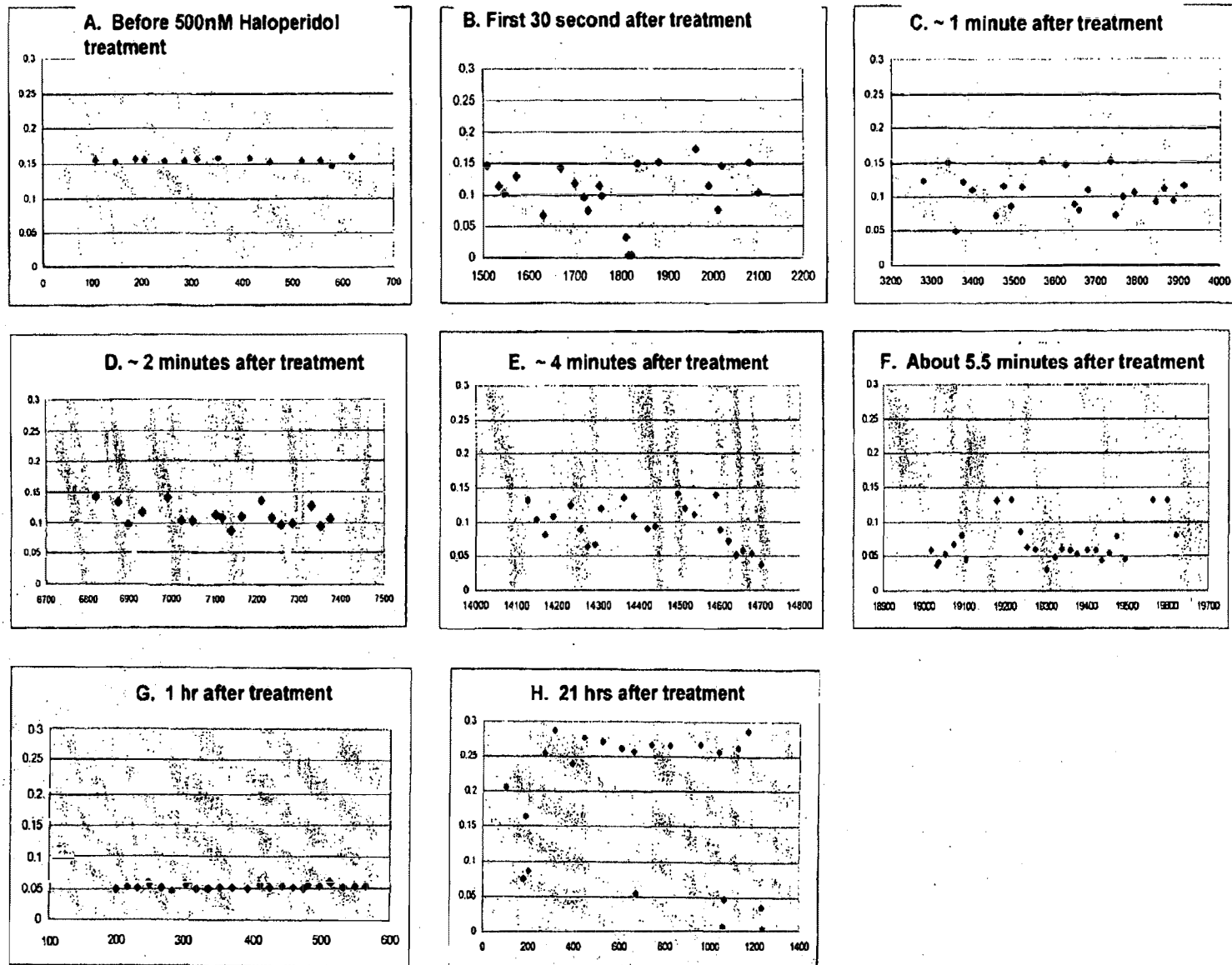


FIG. 17

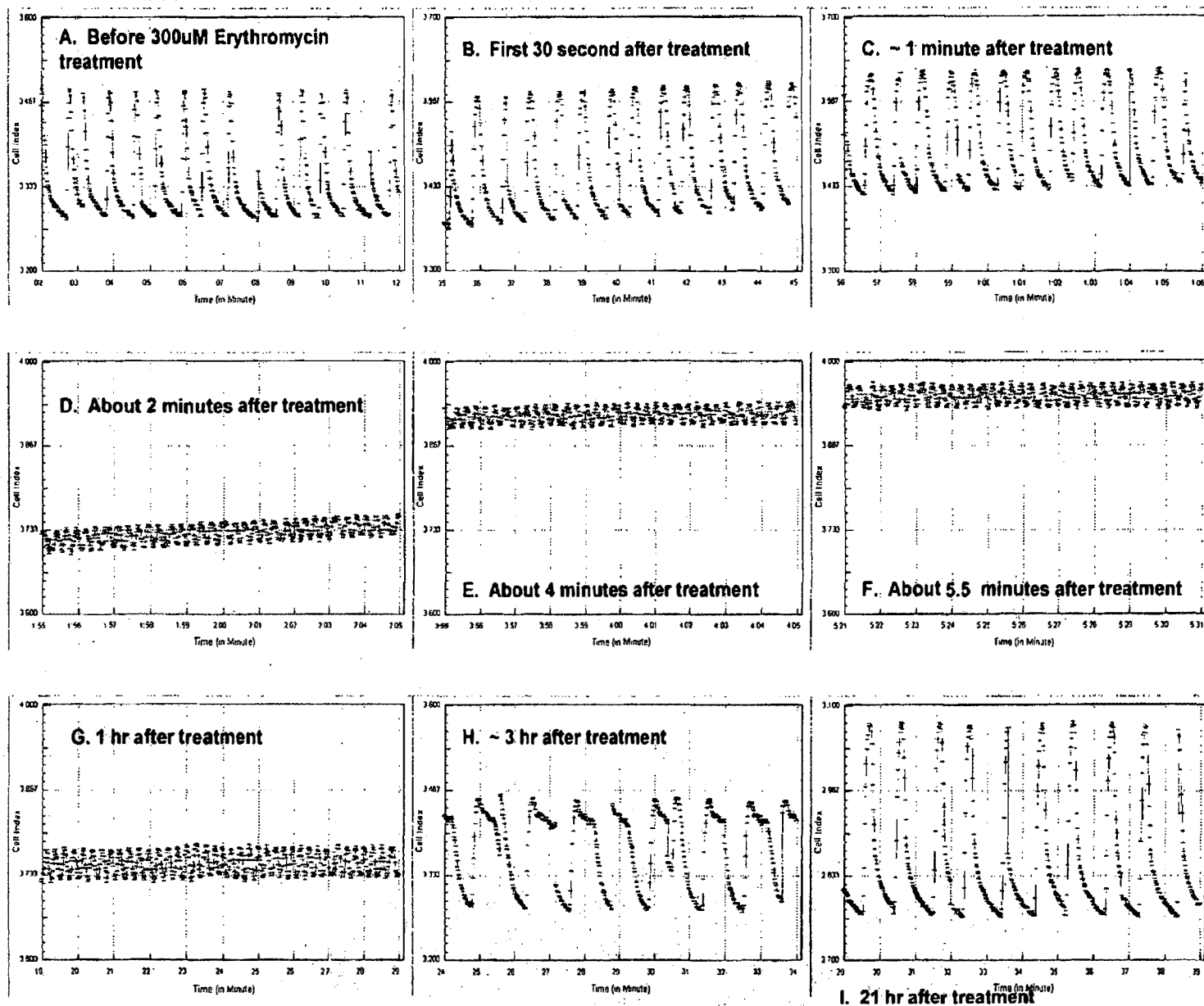


FIG. 18

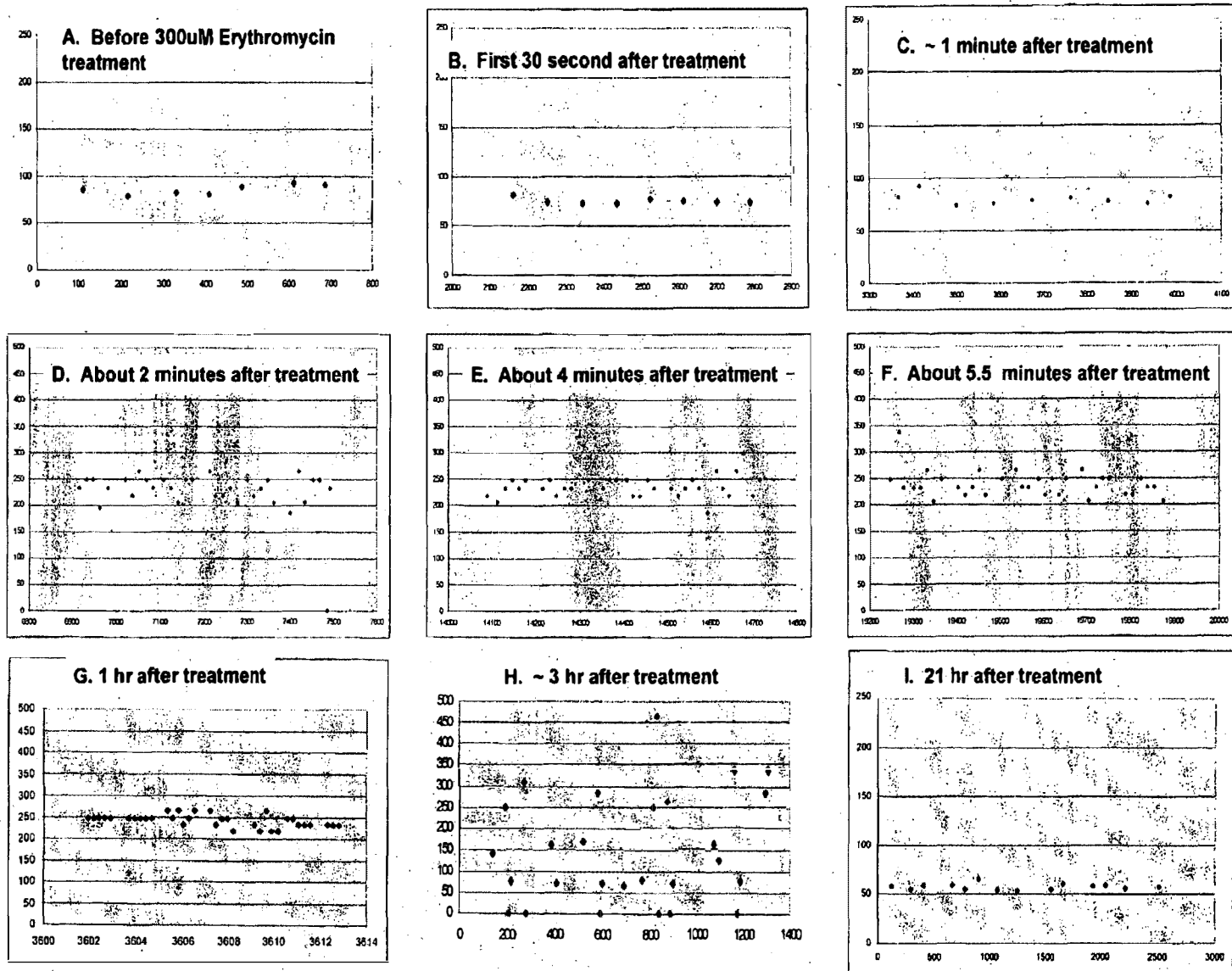
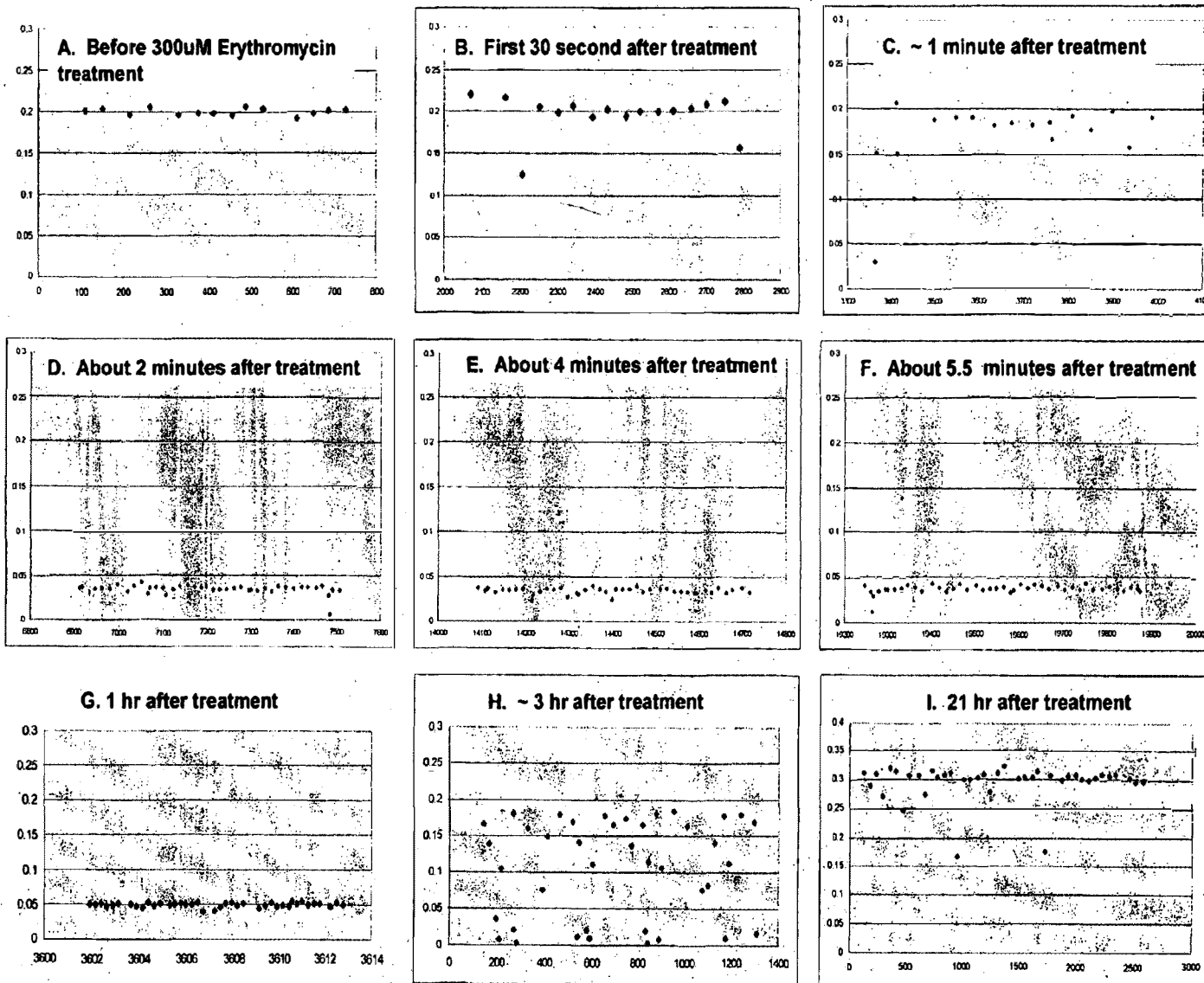


FIG. 19



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- US 11235938 B [0106]

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- **YU et al.** Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Analytical Chemistry*, 2006, vol. 78, 35-43 [0153]

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61/191,684 11 September 2008 (11.09.2008) US

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Suite 100, San Diego, CA 92121 (US). **XI, Biao**
[CN/US]; Acea Biosciences, Inc., 6779 Mesa Ridge Rd.,
Suite 100, San Diego, CA 92121 (US). **ZHANG, Wen,**
Fu [CA/US]; Acea Biosciences, Inc., 6779 Mesa Ridge
Rd., Suite 100, San Diego, CA 92121 (US). **XU, Xiao**
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EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
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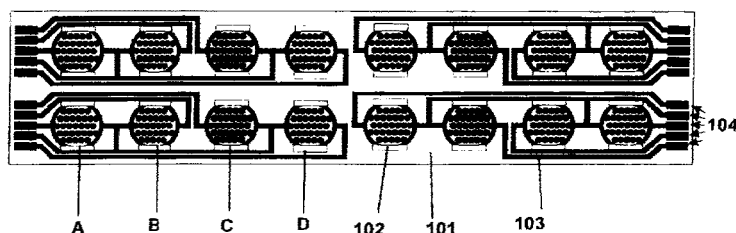


FIG. 1A

(57) Abstract: Systems and methods for improved monitoring of excitation-contraction coupling and excitable cells are provided, which provide millisecond time resolution. The system is capable of continuously monitoring excitation-contraction coupling in a relatively high-throughput manner. The system includes a device for monitoring cell-substrate 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 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.

**LABEL-FREE MONITORING OF EXCITATION-CONTRACTION
COUPLING AND EXCITABLE CELLS USING IMPEDANCE BASED
SYSTEMS WITH MILLISECOND TIME RESOLUTION**

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority to U.S. provisional patent application serial no. 61/191,684, filed on September 11, 2008, and U.S. provisional patent application serial no. 61/126,533, filed on May 5, 2008, the contents of both
10 are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to impedance based monitoring of cells
15 and more particularly to systems and methods for monitoring excitation-contraction coupling and excitable cells using impedance based systems with millisecond time resolution.

BACKGROUND OF THE INVENTION

20 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 contraction. Although ECC has
25 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
30 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 patch clamping in an array format, which even though is becoming increasingly available, is not yet widespread. In addition, cardiac toxicity may occur by other mechanisms that may be missed by this type of targeted approach.

An alternative to 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 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 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 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 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.

5 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

10 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 systems typically only monitor a single cardiomyocyte or a small number of

15 cardiomyocytes at a time and with very limited throughput.

SUMMARY OF THE INVENTION

The present invention addresses the need to provide systems and methods for

20 improved monitoring of excitation-contraction coupling and excitable cells and provides related benefits. Specifically, embodiments of the invention describe label-free methods for monitoring excitable cells, such as cardiomyocytes, in vitro. The system is capable of continuously monitoring excitation-contraction coupling in a relatively high-throughput manner. The systems and methods can be used for

25 pharmacological safety assessment, screening for novel compounds which modulate cardiomyocyte function in a specific manner, and assessment of genes involved or potentially involved in cardiac function. In addition the system and methods may study the role of different genes and proteins in cardiac function and development through the use of embryonic stem (ES) derived cardiomyocytes.

30 In one aspect of the present invention a system for monitoring impedance of excitable cells in vitro is provided including a device for monitoring cell-substrate 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 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. Embodiments of the device for monitoring substrate impedance include a nonconductive substrate having one or more individually addressable electrode arrays fabricated thereon and one or more wells. Preferably, each of the one or more arrays is associated with one of the one or more wells and each electrode array is individually addressable. In further embodiments, a surface of the substrate is suitable for cell attachment, wherein cell attachment results in a detectable change in impedance between electrodes within the array. Attachment or growth along the surface may be enhanced by applying a precoat having one or more compounds that improve attachment. In embodiments where the device includes multiple wells, the impedance analyzer has the capability to allow impedance measurements for each and every well at millisecond time resolution. That is to say, for a selected time period, the impedance data for each and every well of the system is measured at millisecond time resolution. Further, when groups of wells are monitored, the groups are monitored at millisecond time resolution.

Embodiments of the system include electrode arrays having two electrode structures that have substantially the same surface area. The electrodes themselves may be formed from suitable electrically conductive materials such as metal, gold, platinum, titanium, chromium and the like. The electrode arrays may be organized in a variety of configurations, including interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal, and castellated. The widths of the electrode structures may be between 20 microns and 500 microns or 5 microns and 100 microns.

Embodiments of the invention include impedance monitoring using millisecond time resolution. Millisecond time resolution allows monitoring of at least two consecutive impedance measurements with an excitation cycle of excitable cells, which are introduced into the well. Further, millisecond time resolution enhances measurement such that excitation-contraction coupling of excitable cells may be effectively monitored. In some embodiments the excitation cycle is a beating cycle, such as a beating cycle of a cardiomyocyte. In some embodiments at least two consecutive impedance measurements are performed in less than 100 milliseconds. In

other embodiments at least two consecutive impedance measurements are performed in less than 40 milliseconds. In still further embodiments at least two consecutive impedance measurements are performed in less than 20 milliseconds. In still further embodiments the at least two consecutive impedance measurements are performed in
5 less 10 milliseconds.

In some embodiments the device is provided as a multiwell device or a multiwell plate. In some embodiments, the multiwell device or multiwell plate may including 6 wells, 8 wells, 16 wells, 32 wells, 96 wells, 384 wells or any number therebetween. Throughout the present application where the device includes multiple
10 wells each of which is associated with an electrode array, millisecond time resolution refers to that for any selected time period, the impedance measurement for each and every well can be performed with two consecutive measurements separated with millisecond resolution. Further, when groups of wells, such as subsets of all wells of a device, are monitored a group of wells may be monitored using millisecond time
15 resolution.

Embodiments of the present invention provide impedance-based monitoring with millisecond time resolution in combination with longer term monitoring of cells. By combining millisecond time resolution technology with impedance monitoring having longer time periods, both short term and long term effects on excitable cells
20 can be evaluated. In some embodiments impedance readouts can be used to monitor both short term beating and long term viability status of cardiomyocytes in the same well or the same population. Such monitoring addresses needs where certain manipulations such as drug treatment may not manifest its effect on cardiomyocyte beating and/or viability until a later time period.

In some embodiments methods for assessing and quantifying excitable cells in vitro include providing the impedance-based system with millisecond time resolution; adding excitable cells to one or more wells; monitoring impedance of the one or more wells over a first time period, which is characterized as a longer time period, such as seconds, minutes, hours or days; monitoring impedance of the one or more wells over
30 a second time period in millisecond time resolution; determining a characteristic, such as cell attachment, cell growth and cell viability from monitoring over the first time period; and resolving individual cycles of the excitable cells from monitoring over the second time period. In such embodiments, monitoring impedance over the second

time period may be performed at regular or irregular time intervals within monitoring impedance over the first time period.

Among the benefits, embodiments of the invention permit impedance readouts to monitor the morphological or differentiative behavior of cardiomyocytes in vitro.

- 5 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.

- 10 In another aspect of the present invention a method for assessing or quantifying excitable cells in vitro is provided. An exemplary method includes providing the impedance based system having millisecond time resolution, adding excitable cells to the one or more wells, monitoring impedance of the one or more wells, and resolving individual cycles of the excitable cells. The method has particular utility with cells that are known or suspected to be capable of or undergo
15 excitation-contraction coupling, such as cardiomyocytes. The method may also be useful for monitoring neurological cells or neurological systems. Impedance measurements may be performed in millisecond time resolution. In some embodiments, two consecutive impedance measurements for each and every well of the system are performed less than 300 milliseconds apart. In further embodiments, at
20 least two consecutive impedance measurements for each and every well of the system are performed less than 100 milliseconds apart. In further embodiments, at least two consecutive impedance measurements for each and every well of the system are performed less than 40 milliseconds apart. In further embodiments, at least two consecutive impedance measurements for each and every well of the system are less
25 than 10 milliseconds apart.

- In some embodiments methods of determining whether a compound modulates a beating cycle of an excitable cell is provided. An exemplary method includes providing the impedance based system having millisecond time resolution, wherein the system includes at least two wells; adding excitable cells to the at least
30 two wells; monitoring impedance of the at least two wells; adding a compound suspected of modulation of the beating cycle to a first of the at least two wells to provide a test well, wherein a second well lacking compound or having a control compound is provided as a control well; resolving the beating cycle of both test well

and control well; and comparing the beating cycles between the test well and control well, wherein a difference in beating cycles indicates the compound modulates the beating cycle of the excitable cell. In some embodiments, beating cycles are compared to identify changes in beating magnitude or amplitude. In other
5 embodiments, beating cycles are compared to identify changes in beating frequency, which may be regular changes or irregular changes. In further embodiments the method includes determining a cell index for each of the test well and control well such that the step of comparing the beating cycles includes comparing the cell indices between test well and control well. Peak magnitude or amplitude and/or frequency
10 may be compared. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance monitoring with intermittent monitoring using millisecond resolution. Thus short term and long term monitoring may be performed.

In another exemplary methods include providing a device for measuring cell-
15 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
20 impedance-based curve or optionally a cell index curve for each of the at least one known biologically active agent 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 may be direct measurement of cardiomyocyte excitation-contraction coupling and if significantly
25 different, concluding that the biologically active agent modulates cardiomyocyte function. 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
30 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.

In other embodiments of the present invention, a method of characterizing rhythmic beating of a cardiomyocyte is provided. An exemplary method includes providing the impedance based system with millisecond time resolution; adding excitable cells to the one or more wells; monitoring impedance of the one or more wells in millisecond time resolution; determining a plurality of beating cycle peaks; and comparing magnitude or amplitude or frequency of the peaks over a time unit. Various methods can be used for determining beating cycle peaks. For 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.

10 The beating cycle peaks are those data points where the first order derivatives of impedance values or cell index values are zero or close to zero in its 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 the data points where the second order derivatives of the impedance

15 values or cell index values is negative and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its 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

20 values or cell index values is positive and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its absolute value. The step of determining a plurality of beating cycle peaks may include determining a rise in impedance and a decay in impedance and extrapolating a peak from each rise and decay. The method may further include determining a cell index for each of the

25 one or more wells, which may allow improved comparison of beating cycles. Further, beating cycle peaks may be determined by identifying frequency components having largest magnitude in Fourier transformed impedance data. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance monitoring with intermittent monitoring using

30 millisecond resolution.

A variety of agents and compounds may be tested for modulation of excitable cells and for modulation of rhythmic beating of cardiomyocytes. Examples include

compounds, peptides, proteins, antibodies or antibody fragments, siRNA, shRNA, lipid or any combination of thereof.

In another aspect of the present invention a method of assessing genetic manipulation of an embryonic stem cell is provided including providing the
5 impedance based system having millisecond time resolution, mutating embryonic stem cells to include a genetic mutation; adding mutated embryonic stem cells to a first well of at least two wells to form a test well and control embryonic stem cells to a second of at least two wells to form a control well; monitoring impedance of the at least two wells in millisecond time resolution; resolving the beating cycles of both test
10 well and control well, if any; and comparing the beating cycles between the test well and control well, wherein a difference in beating cycles indicates the genetic mutation modulates embryonic stem cell differentiation. In some embodiments the embryonic stem cell differentiation is differentiation into a cardiomyocyte. In some embodiments the genetic mutation is selected from the group consisting of a genetic
15 knock out, a knock down and an inserted transgene. The method may also include monitoring impedance over longer time periods of time. Such embodiments may provide long term impedance monitoring with intermittent monitoring using millisecond resolution. Thus short term and long term monitoring may be performed.

BRIEF DESCRIPTION OF THE DRAWINGS

Those skilled in the art will recognize that the drawings described below are for illustrative purposes only. The drawings are not intended to limit the scope of the invention but to provide exemplary embodiments.

FIG.1 depicts schematic drawings of one design of a cell-substrate impedance measurement device of the present invention. **FIG. 1A** shows a nonconductive substrate (**101**) shown with 16 electrode arrays fabricated on the substrate. Each electrode array (**102**) comprises two electrode structures (shown in detail in **FIG. 1B**). Each electrode structure comprises multiple electrode elements. Each electrode array
30 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 the four electrode arrays in each of four quarters on the substrate (101) have one of their electrical connection traces (103) connected to a common connection pad (104). Thus, for the entire 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). In **FIG. 1B** a single exemplary electrode array 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 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. 2A is a schematic representation of a device 100 with two electrode structures of same or similar areas deposited on a substrate. First electrode structure has electrode elements 110a, 110b, 110c and second electrode structure has electrode elements 120a, 120b, 120c and 120d. Electrode elements within an electrode structure are connected to each other by arc-shaped connection electrode bus 125. Like the electrode elements, such connection-buses (125) are also made of electrically-conductive material (e.g. gold film, platinum film, gold film over a chromium or titanium film). These electrically-conductive connection-paths or connection buses (125) may have an insulating coating. Electrode elements 110a-110c and 120a-120d comprise electrode lines with connected circles added on the line. The overall area of electrode elements and gaps between electrode elements may correspond to, or may be slightly larger than, or may be slightly smaller than, the bottom of a well (e.g., a cylinder shaped well, a conical shaped well, or a cubic shaped well), for example, a 24 well-plate, a 96-well plate, or 384 well plate that are commonly used. The whole surfaces of the wells may be covered with electrodes to

ensure that the cell attachment at nearly any locations of the bottom surface of the well can contribute to the impedance change. This arrangement has an advantage that non-uniform landing and attachment of cells on the bottom surface of the different wells would result in only a small variation in the impedance measured between electrode structures **110** and **120**. **150** are connection pads that can be connected to an external impedance measurement circuit. **130** is the electrical connection traces that connects the connection pad to the electrode structures **110** and **120**. Such connection traces can extend in any direction in the plane of the electrodes. **FIG. 2B** is a schematic representation of a device **200** with two electrode structures of similar areas deposited on a substrate. Electrode structures **210** and **220** comprise multiple interconnected electrode elements. Electrode elements (**210a-210c**, **220a-220d**) are rectangular lines and together form an interdigitated electrode structure unit. Similar to **FIG. 2A**, the electrode elements (**210a-210c**, **220a-220d**) within each electrode structure are connected through arc-shaped, electrically conductive paths or electrode buses (**225**). Connection pads **250** are connected to electrode structures through the electrical connection traces **230**. **FIG. 2C** is a schematic representation of a device **300** with two electrode structures of similar areas deposited on a substrate. Electrode structures **310** and **320** comprise multiple interconnected electrode elements (**310a-310f**, **320a-320f**). Electrode elements (**310a-310c**, **320a-320d**) are rectangular lines and together form an interdigitated electrode structure unit. Different from **FIG. 2A** and **FIG. 2B**, the electrode structures having electrode elements **310a-310c** and **320a-320c** are connected to connection pads **350**.

FIG. 3 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. In this example, the impedance measurement was taken with a time resolution of 1 hour between any two consecutive data points for each well.

FIG. 4A provides a plot of normalized cell index values for cells in different wells treated with different concentrations of sodium dichromate dehydrate (SDD). **FIG. 4B** provides the corresponding sigmoidal curve for deriving the IC-50 of the compound.

FIG. 5A provides a graph of normalized impedance data over time showing shifts in morphology in response to isoproteranol, a β_2 adrenergic receptor agonist.

FIG. 5B shows a plot of the normalized cell indices against the log of the corresponding isoproteranol concentrations to calculate the EC-50.

FIG. 6 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 long term measurement, cell electrode impedance and corresponding cell indices were measured at about 15 minute intervals. Thus for each well, the impedance measurement was performed with a time resolution of 15 minutes.

FIGS. 7A-D provides a series of graphs showing cardiomyocyte beating throughout attachment and growth phases.

FIG. 8A and **FIG. 8B** are a pair of cell index curves and the corresponding beat rate derived from Fourier transformation of impedance values

FIGS. 9A-D show cardiomyocyte beating data from mouse cardiomyocytes treated with sotalol. In **FIGS. 9C** and **9D**, the time resolution between two adjacent points is 40 milliseconds. In other words, a second in **FIGS. 7C** and **7D** is equivalent to 40 milliseconds.

FIG 10A depicts graphs of agents which slow down heart rate and cardiomyocyte beating. **FIG. 10B** depicts graphs of agents which increase heart rate and cardiomyocyte beating.

FIGS. 11A-S depict impedance data of cardiomyocyte patterns associated with testing of a variety of known compounds, which are further described in **Table II**.

FIG. 12 depicts impedance based cardiotoxic patterns from known compounds.

FIG. 13 shows an example of dose dependent effects of Astemizole on cardiomyocytes beating at different concentrations.

FIGS. 14A - H are graphical depictions of real time impedance-based millisecond time resolution of cardiomyocyte beating in response to Haloperidol treatment. The Y-axis is the cell index value.

FIGS. 15A-H depict graphical analysis of the impedance beating frequency from impedance plots (cell index plots) shown in **FIGS. 14A – H**.

FIGS. 16A-H depict additional graphical analysis of the magnitude or amplitude of the beating cycle peaks from impedance plots (cell index plots) shown in **FIGS. 14A-H**.

5 **FIGS. 17A-I** are graphical depictions of real time impedance-based millisecond time resolution of cardiomyocyte beating in response to Erythromycin treatment. The Y-axis is the cell index value.

FIGS. 18A-I depict graphical analysis of the impedance beating frequency from impedance plots (cell index plots) shown in **FIGS. 17A – I**.

10 **FIGS. 19A-I** depict additional graphical analysis of beating magnitude or amplitude of the beating cycle peaks shown from impedance plots (cell index plots) shown in **FIGS. 17A – I**.

DETAILED DESCRIPTION OF THE INVENTION

15 Detailed descriptions of preferred embodiments of the provided herein. It is to be understood; however, that the present invention may be embodied in various forms. Therefore specific reference to various forms are provided as a basis for the claims and for teaching one skilled in the present art to employ the present invention in appropriate system, structure or manner.

20

A. Definitions

Unless defined 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
25 other publications referred to herein are incorporated by reference in their entirety. If a definition set 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.

30 As used herein, “a” or “an” means “at least one” or “one or more.”

As used herein, “membrane” is a sheet of material.

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

5 As used herein, “suitable for attachment” refers to structural features that permit attachment of cells thereto. A surface is “suitable for cell attachment” when a significant percentage of the cells attach or adhere to the surface within twelve hours. Preferably, at least 50% of the cells are adhered to the surface within twelve hours. More preferably, at least 70% of the cells are adhered to the surface within twelve
10 hours of plating (i.e., adding cells to the well or fluid container). 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 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,
15 four, two hours of plating. To have desired surface properties for cell attachment, the surface may need to be chemically-treated (e.g. treatment with an acid and/or with a base), and/or physically treated (e.g. treatment with plasma), and/or biochemically treated (e.g. coated with one or more molecules or biomolecules that promotes cell attachment).

20 As used herein, “impedance measurement at millisecond time resolution” refers to the ability to perform a series of at least two consecutive impedance measurements within milliseconds of one another for each and every well of the system where each well is associated with an electrode array for impedance measurement. In each instance the time between two consecutive impedance
25 measurements are less than 300 milliseconds from one another. Preferably, consecutive impedance measurements are less than 200 milliseconds from one another. More preferably impedance measurements are less than 100 milliseconds from one another. For example, the time between two consecutive impedance measurements are 40 milliseconds from one another for each and every well of the
30 system. To be clear, that is to say, in a time period of 40 milliseconds, the system has the ability to measure two impedance data points for each and every well of the system.

As used herein, “excitable cells” refers to a cell population that is capable of propagation and spreading action potentials to surrounding cells. These cells are able to produce and respond to electrical signals. Some excitable cells are capable of excitation-contraction coupling. Examples of “excitable cells” include

5 cardiomyocytes, cells that beat, some skeletal muscle cells, some smooth muscle cells and some neural cells. Identification of excitable cells may be found in cell biology literature.

As used herein, “excitation-contraction coupling” refers to the physiological process of converting an electrical stimulus to mechanical response. This process is

10 fundamental to muscle physiology, whereby the electrical stimulus is usually an action potential and the mechanical response is contraction. EC coupling can be dysregulated in many disease conditions.

As used herein, “beating cycle” refers the time between which a cardiomyocyte sequentially beats. The “beating cycle” may vary depending on

15 organism and developmental stage of the cardiomyocyte. A beating cycle may be as short as between 100 and 200 milliseconds, translating to a beating rate of 600 to 300 beats per minute. A beating cycle may be as long as between 1000 milliseconds and 10,000 milliseconds, translating to a beating rate of 60 to 6 beats per minute.

As used herein, “biomolecular coating” or “coated with a biomolecule” refers

20 to is a coating on a surface that comprises a molecule that is a naturally occurring biomolecule or biochemical, or a biochemical derived from or based on one or more naturally occurring biomolecules or biochemicals. For example, a biomolecular coating can comprise an extracellular matrix component (e.g., fibronectin, collagens), or a derivative thereof, or can comprise a biochemical such as polylysine or

25 polyornithine, 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.

As used herein, “extracellular matrix component” refers to a molecule that

30 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.

As used herein, “electrode” refers to a structure having a high electrical conductivity, that is, an electrical conductivity much higher than the electrical
5 conductivity of the surrounding materials.

As used herein “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.

10 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.

As used herein “electrode structure unit” refers to two or more electrode structures that are constructed to have dimensions and spacing such that they can,
15 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.

20 As used herein, “electrode traces” refer to 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 impedance analyzer. The end or boundary of a device may correspond to the connection pads on the device or apparatus.

25 As used herein, “connection pad” refers to 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). The electrical connection
30 between a connection pad and an impedance measurement 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.

As used herein, “interdigitated” refers to having projections coming from 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).

As used herein, “high probability of contacting an electrode element” refers to 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 on 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%.

As used herein, “at least two electrodes fabricated on said substrate” refers to 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.

As used herein, “at least two electrodes fabricated to a same side of said substrate” refers to at least two electrodes are fabricated on the same side of the substrate.

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

As used herein, “electrodes have substantially same surface area” refers to 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 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 referred to. In other words, where electrodes 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 structure is less than 5, 4, 3, 2, 1.5, 1.2 or 1.1. More preferably, the at least two electrodes of an electrode structure have nearly identical or identical surface area.

As used herein, “said device has a surface suitable for cell attachment or growth” refers to 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 necessary substances, e.g., nutrients or growth factors, can be supplied in a medium. Preferably, when a suspension of viable, unimpaired, excitable cells is added to the “surface suitable for cell attachment” at least 50% of the cells adhere to the surface within twelve hours. More preferably, a surface that is 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 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 the electrodes” refers to the impedance between or among the electrodes would have a significant change that can be detected by an impedance analyzer or impedance measurement circuit when molecule binding reaction or cell attachment or cell adhesion or cell presence occurs on the electrode surfaces. The impedance change refers to the difference in impedance values when cell attachment or cell adhesion or cell presence or molecule binding reaction occurs on the electrode surface of the apparatus and when no molecular reaction occurs on the electrode surface or no cell is present on the electrode surface. The impedance change may occur upon the presence or decay of a beat from a cardiomyocyte. Alternatively, 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 surface, or when the number, type, activity, 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 the 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 the 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 the 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 the 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” refers to 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” refers to 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.

As used herein, “each well contains substantially same number . . . of cells” refers to 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 well contains an identical number of cells.

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 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. In some instances the “same type of cells” may initially refer to embryonic stem cells; however, the stage of differentiation into cardiomyocytes may be differ.

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.

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 be cardiotoxic if it impairs cardiomyocyte beating, whether magnitude or

amplitude or frequency, or if it causes cell death. Then the response of cells can be measured by percentage of non-viable (or viable) cardiac cells after the cells are treated by the test compound.

As used herein, "microelectrode strip or electrode strip" refers to a non-
5 conducting substrate strip on which electrodes or electrode structure units are fabricated or incorporated. The non-limiting examples of the non-conducting substrate strips 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
10 substrate strip by any suitable microfabrication, micromachining, or other methods. Non-limiting examples of electrode geometries include interdigitated electrodes, circle-on-line electrodes, diamond-on-line electrodes, castellated 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
15 200 micron, over 500 micron, over 1 mm. The characteristic dimensions of the electrode geometries refer to the smallest width of the electrode elements, or 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
20 – 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
25 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-
30 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.

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
5 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, modulating the beating cycle of a cardiomyocyte cell population, whether by beating amplitude or frequency. In still another application, a
10 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
15 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 cardiotoxicity or embryonic stem cell development, etc.

20 An "impedance value" is the impedance measured for electrodes in a well with or without cell 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
25 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
30 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.

5 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.

10 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 derive of the Cell Index with respect to time, divided by the Cell Index at the time point. In other words, CCI is calculated as

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

15

As used herein, "primary cell" or "primary cells" refers to any non-immortalized cell that has been derived from various tissues and organs of a patient or an animal.

20 B. Introduction to Impedance-Based Systems and Methods having Millisecond Time Resolution

25 The impedance-based system and methods of the present invention fill a major technological gap in monitoring of excitation-contraction coupling and excitable cells, such as cardiomyocytes, in vitro. This is accomplished, in part, from the development of an impedance-based system and monitoring method that employs millisecond time resolution. Millisecond timer resolution permits effective monitoring of excitable cells for short durations, such as less than about 100 milliseconds. Further by coupling millisecond time resolution with longer duration
30 time periods, both short term and long term effects of drugs, such as cell viability, changes in morphology and adhesion can also be assessed. These combination studies

permit further data collection with respect to compounds such as β -2 adrenergic receptor agonists, well known and characterized modulators of heart function in vivo and in vitro, which can induce long term hypertrophic responses in cardiomyocytes, which is associated with elongated morphology of the cells. When combining long term monitoring and short term monitoring it may be desirable to provide long term monitoring coupled to intermittent short term monitoring periods.

The devices, systems and methods provided permit impedance-based monitoring of excitable cells using millisecond time resolution. It has been discovered that by developing enhanced impedance based systems, detailed analysis of excitable cells, the excitation cycle of cells and potential modulators of excitable cells can be performed. Further, by studying the excitation cycle with increased resolution embodiments of the invention permit the effective evaluation of factors that may alter normal or abnormal cycling. Without millisecond time resolution, it would not be possible to perform detailed analysis of the beating cycle of the excitable cells. For example, for cardiomyocytes, if the impedance is measured at seconds time-resolution such as a ten-second or even one-second resolution, it would not be possible to observe beating of the cardiomyocytes or resolve a beating cycle of the cardiomyocytes with an impedance measurement. The cardiomyocytes-beating-resulted impedance change would be shown as "noise" on the impedance curves measured at seconds or minutes time-resolution. Indeed, it was a surprise that the beating of the cardiomyocytes on the electrode surfaces would result in periodic change in impedance and even more surprise that the impedance measurement, when performed at appropriate time resolution of milli-seconds, could resolve such beating cycles. After we discovered that cardiomyocyte beating, when cultured on electrode surfaces, can be monitored via impedance measurement, we interpreted that such dynamic monitoring of cardiomyocyte beating is based on quantification in real time of the rhythmic changes in cardiomyocyte morphology and/or attachment as a result of the excitation contraction coupling of the electrically excitable cardiomyocytes growing on microelectrodes' surface in E-PLATES (ACEA Biosciences, San Diego, CA). The quantification of the rhythmic changes in cardiomyocyte morphology and/or attachment is achieved via the fast (millisecond resolution) 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.

When combining long term impedance monitoring and short term monitoring it may be desirable to provide long term monitoring coupled to intermittent short term monitoring. In other words, while long term impedance monitoring is able to identify long term effects on excitable cells, short term impedance monitoring, such as at millisecond time resolution may be employed at particular time points to assess short term effects. In some instances, impedance monitoring with millisecond time resolution is performed at regular or irregular time intervals. In these instances, time intervals may be preprogrammed in the system or may be initiated by the user. In some instances intermittent measuring at millisecond time resolution is initiated upon the occurrence of an event, such as an event detected or determined while long term monitoring of impedance. Such events may be changes in measured impedance data, changes in optical property and the like. Monitoring at millisecond time resolution may continue until a programmed event occurs, such as expiration of a time period. After or during which, long term impedance monitoring may continue.

In some embodiments, long term and short term monitoring of excitation-contraction coupling or excitable cells is provided in combination. Exemplary methods include providing the impedance-based system with millisecond time resolution; adding excitable cells or cells suspected of excitation-contraction coupling to one or more wells; monitoring impedance of the one or more wells over a first time period, which is characterized as a longer time period, such as seconds, minutes, hours or days; monitoring impedance of the one or more wells over a second time period in millisecond time resolution; determining a characteristic, such as cell attachment, cell growth and cell viability from monitoring over the first time period; and resolving individual cycles of the excitable cells from monitoring over the second time period. In such embodiments, monitoring impedance over the second time period may be performed at regular or irregular time intervals within monitoring impedance over the first time period. Further, test agents may be added to assess their short term effects and long term effects on the cells.

Impedance monitoring using, at least in part, millisecond time resolution will be useful in the testing of compounds such as drugs for the treatment or prevention of various medical conditions or their safety. In some embodiments the systems and

methods are used to test or evaluate potential compounds, drugs or treatments for cardiotoxicity data. In some embodiments potential compounds, drugs or treatments are tested for cardioprotective data. In some embodiments potential compounds, drugs or treatments are tested for potential applicability for cardiovascular treatments, including hypertension or congestive heart failure. In some embodiments potential compounds, drugs or treatments are tested for modulation of embryonic stem cell development, such as development into cardiomyocytes. In other embodiments the systems and methods are used to test or assess potential drugs or treatments for neurological data, such as beneficial or deleterious neurological effects. As will be understood by those skilled in the present art, the impedance based system and methods including millisecond time resolution permits evaluation across multiple cell populations or systems that including excitation-contraction coupling.

One skilled in the art will understand embodiments of the present invention may be used for studying a variety of excitable cells or effects thereon. As nonlimiting examples cells may be cardiac muscle cells, neurological cells, differentiating stem cells, embryonic stem cells, adult stem cells and the like. Smooth muscle cells and skeletal muscle cells that undergo excitation-contraction coupling may also be studied.

In some embodiments of the present invention the excitation cycle or beating of cardiomyocytes is studied. Cardiomyocytes are specialized muscle cells, which make up the majority of the volume of the myocardium. The myocardium also includes epithelial cells and fibroblasts. Cardiomyocytes are arranged largely in a circumferential and spiral orientation around the left ventricle, the chamber that pumps blood to the systemic circulation. Atrial myocytes, also cardiomyocytes, are smaller in diameter and less structured than their ventricular counterparts. Cardiomyocytes have five major components: cell membrane (sarcolemma) and T-tubules, for impulse conduction; sarcoplasmic reticulum, a calcium reservoir needed for contraction; contractile elements; mitochondria; and nucleus. Cellularly, cardiomyocytes may be identified by the presence of cardiac troponin T (TnT) and atrial natriuretic peptide (ANP).

The functional intracellular contractile unit of cardiac muscle (like skeletal muscle) is the sarcomere, an orderly arrangement of thick filaments composed principally of myosin, and thin filaments containing actin. Contraction of cardiac

muscle occurs by the cumulative effort of sliding of the actin filaments between the myosin filaments toward the center of each sarcomere.

Cardiomyocytes may be obtained or isolated from cardiac tissue directly, may be cultured from cardiomyocyte cells or may be differentiated from stem cells, such as embryonic stem cells. In some embodiments embryonic or adult stem cells are used. Methods for obtaining or isolating cardiomyocytes from cardiac tissue may be found in the literature.

Since embryonic stem (ES) cells are self renewing cells in culture they can serve as an excellent source for continuous production of cardiomyocytes. These derived 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. 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 concern of adverse affects on overall embryonic development in transgenic animals. Further, the ability to 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 express the GFP transgenes and can be easily identified and isolated by cell sorting techniques and therefore an enriched cardiomyocyte population can be obtained.

C. Impedance-Based Systems Having Millisecond Time Resolution

In preferred embodiments the system for monitoring impedance of excitable cells in vitro includes a device for monitoring cell-substrate 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 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 impedance based system with millisecond time resolution, excitation-contraction coupling cells can be efficiently monitored and studied. Accordingly, the systems provided herein are able to identify and evaluate changes in excitation-contraction events, which may be used for high throughput analysis of potential therapeutics. 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.

Embodiments of the device for monitoring substrate impedance include a nonconductive substrate having one or more individually addressable electrode arrays 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 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 and urea resin. Preferably, the substrate is biocompatible with excitable cells; 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 precoating the substrate with a protein or compound that facilitates 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.

Each electrode array includes 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. 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 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 by 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 does 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 does 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 does 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 does 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.

Descriptions of electrode arrays used for impedance measurement that apply to the devices of the present invention are 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.

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 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 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, 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.

A device of the present invention can include one or more fluid-impermeable receptacles which serve as fluid containers or wells. Such receptacles may be
5 reversibly or 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
10 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 Application 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
15 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
20 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-
25 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
30 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 excitable 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 1Hz 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 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 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 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 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 impedance measurements between 50 milliseconds and 100 milliseconds apart. In some instances millisecond time resolution includes at least two consecutive
5 impedance measurements between 100 milliseconds and 150 milliseconds or between 150 and 300 milliseconds apart.

Millisecond time resolution of impedance-based devices provides a significant technical step over traditional impedance measuring devices, which are typically limited to second or minute resolution. That is, substantial reconfiguration of a
10 traditional impedance-based system is required to obtain millisecond time resolution. Advances in multiple steps are required to achieve millisecond time resolution, including the impedance measurement circuitry together with electronic switching circuitry. Technological advances are further required when performing millisecond time resolution over multiple wells, such as 4 wells, 8 wells, 16 wells or 96 wells.
15 For example, if the system comprises a device having 96 wells, the system hardware and software should be capable of, for a given time period, measuring the impedances of all 96 wells with millisecond time resolution between two adjacent or consecutive impedance measurement points for each and every well. Thus, comparing two impedance analyzers, one for impedance measurement of 96 wells with millisecond
20 resolution and another for impedance measurement of a single well with millisecond time resolution, the one for 96 well measurement is effectively 96-times faster than one for a single well measurement since it is required to have a capability for performing impedance measurement for all 96 wells with millisecond time resolution. Similarly, if a 96 well device is provided, a group of wells, such as a group of 2, 4, 6,
25 12, 24 or 48 wells, may be measured in millisecond time resolution. 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. Still more preferably, the time difference between two adjacent
30 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.

A number of improvements in the impedance measurement circuitry,
5 electronic switching circuitry, communication between impedance measurement circuitry and software can be used to achieve such millisecond time resolution. For instance, improvement likely requires the use of fast processing electronic chips for analogue-to-digital conversion, for parallel digital signal processing and data
10 calculation with field-programmable gate array (FPGA) and for fast communication between the impedance measurement circuitry and software. Another example 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 simultaneously. Such parallel AD conversion is important, particular
15 for the system having multiple wells, each of which's measurement time resolution is required to be in the millisecond resolution. And, a very important is to replace previous working mode of "measurement of one-well's impedance at a time" with a mode of "measurement of multiple-wells' impedances at a time". In "one-well at a time" mode, when the software issue a command for measuring one well's
20 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 the communication line between the impedance measurement circuitry and the computer. The system will not perform any measurement for another well until the
25 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' impedances. The measurement circuitry would simultaneously or nearly simultaneously perform signal conversion, signal processing and impedance calculation for multiple wells. The multiple
30 impedance data for the multiple wells would be sent over the communication lines to the computer sequentially with one well's data at the same time or simultaneously with more than one well's data being sent at a time. In this "measurement of multiple-wells' impedances 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.

With millisecond time resolution for impedance measurement, it becomes
5 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
10 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
15 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 switching between occur between designated groups. In one embodiment of the above system, the system
20 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.

25 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
30 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.

Preferably, the software can also analyze impedance data. In preferred embodiments, the software can calculate a cell index (CI) for one or more time points for one or more wells of the multiwell device. In some preferred embodiments, the software can calculate a cell change index (CCI) from impedance measurements of one or more wells of the multiwell device. The software can preferably generate plots of impedance data and impedance values, such as but not limited to CI or CCI, with respect to time. The software may perform other analysis as well, such as calculate cell number from CI, generate dose-response curves based on impedance data, calculate IC values based on impedance values, and calculate kinetic parameters of the excitation cycle cell based on impedance values and impedance value curves. In some embodiments the beating cycle of a cardiomyocyte population is determined, which may include initiation and decay of individual beats. Peaks may be derived from the detection of vectors associated with initiation of beating or beating decay. Peaks may be derived with other methods. In further embodiments the change in beating cycle of a cardiomyocyte population is determined in response to a stimulus

such as a pharmacological agent. The software of the impedance monitoring system can also store and display analyses of the data, such as calculated impedance values and kinetic parameters derived therefrom. Data can be displayed on a screen, as printed data, or both. Data may be stored on a hard drive for exportation into compatible programs for further analysis or data storage

Further 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 no. 11/235,938, 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 each is briefly summarized.

The cell index obtained for a given well reflects: 1) how many cells are attached to the electrode surfaces in this well, 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 cell index = 0. A higher value of "cell number index" indicates that, for 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 same type of the cells and 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.

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
5 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
10 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
15 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.

Thus CCI is the normalized rate of change in cell index. CCI values can be used to quantify the cell status change. For cells in an exponential growth under
20 regular cell culture condition, the cell index determined by a cell-substrate impedance monitoring system described herein is expected to be a proportionate measure of the cell number in the well since the cell morphology and average extent of cell adhesion to the electrode surfaces among the whole cell population do not exhibit significant changes over time.

After measuring and recording time dependent impedance data for excitable
25 cells cultured on electrodes, various methods can be used for determining beating cycle peaks. For 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
30 where the first order derivatives of impedance values or cell index values are zero or close to zero in its 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 the data points where the second order derivatives of the impedance values or cell index values is negative and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its 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 is positive and where the first order derivatives of the impedance values or cell index values is zero or close to zero in its absolute value. In another example, the 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 are 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. The beating rate can be determined by dividing a unit time (e.g., 1 minute) by the time period between two adjacent peaks. For example, if two adjacent peaks are separated by 500 milliseconds, then the beating rate for this 500 milliseconds can be determined as 120 beats per minute. Another method is simply to count how many peaks in a unit time by software. For example, if there are 2 peaks in one second interval, then the beating rate should be 2 beats per second, or 120 beats per minute.

D. Assessing or Quantifying Excitable Cells, Excitation Contraction-Coupling and Cardiomyocytes In Vitro

In another aspect of the present invention a method for assessing or quantifying excitable cells in vitro is provided. The methods including detecting or monitoring excitable cells using impedance monitoring with millisecond time resolution. In preferred embodiments, the method includes providing the impedance based system having millisecond time resolution system, adding excitable cells to one or more wells of the device, monitoring impedance of the one or more wells with

millisecond time resolution, and resolving individual cycles of the excitable cells. In some embodiments monitoring impedance further includes determining cell indices from impedance values, calculating average rate of excitation events per unit time (such as beats per unit time when using cardiomyocytes), determining amplitude
5 intensity or average amplitude intensity in a unit time, and/or determining the length of time between excitation cycles (or beating cycles). These additional determinations may be compared to further assess excitable cells.

The methods have particular utility with cells that are known or suspected to be capable of or undergo excitation-contraction coupling, such as cardiomyocytes.

10 Thus the methods may monitor, detect or determine excitation contraction-coupling of excitable cells. Further, even minor variations in excitation-contraction coupling may be detected.

Preferably impedance measurements are performed with millisecond time resolution. In some embodiments, two consecutive impedance measurements are
15 performed at less than 300 milliseconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 100 seconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 40 milliseconds apart. In further embodiments, at least two consecutive impedance measurements are performed less than 10 milliseconds apart.
20 In some embodiments at least two impedance measurements are between 1 millisecond and 50 milliseconds apart.

Cardiomyocytes are a subset of cells that are involved in excitation-contraction coupling. Embodiments of the present invention include applications for studying cardiomyocyte activity, function and its modulation. By monitoring the
25 continuous rhythmic beating of cardiomyocytes, methods of the present invention allow the study of cardiomyocytes themselves as well as screening for pharmacological agents which may alter cardiomyocyte rhythmic beating. Thus the methods permit screening of compounds or conditions that affect cardiomyocyte function or induce cardiotoxicity. Specifically, methods may assess or quantify
30 cardiomyocyte beating in vitro using impedance-based technology having millisecond time resolution. In preferred embodiments, the methods are based on quantification in real time 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 E-PLATES (ACEA Biosciences, Inc. San Diego, CA). Thus quantification of the rhythmic changes in cardiomyocyte morphology is achieved via the measurement of electrode impedance, which may be performed with millisecond time resolution. In summary, some methods provide a cellular cardio-
5 gram, which can provide incisive information about the status of cardiomyocytes especially upon treatment with pharmacological agents.

Cardiomyocytes used in the present invention may be any known or experimental cardiomyocyte population. Human cardiomyocytes will be particularly desirable when studying the effect of pharmacological agents for human use; however,
10 cardiomyocytes may also be murine or mouse, rat, rabbit, dog, and the like. Further, the cardiomyocytes may be primary cardiomyocytes isolated from cardiac tissue or may be cultured cardiomyocytes, which are cultured over time. In some embodiments, ES cells destined to differentiate into cardiomyocytes are used. Isolated primary cardiomyocytes as well as ES-derived cardiomyocytes retain the
15 ability to beat in culture. Thus, these cells provide a desirable model system to study cardiomyocyte function in vitro.

Cardiomyocytes have an innate ability to undergo mechanotransduction. In other words, the spontaneous force generated from the beating cardiomyocyte is translated to intracellular biochemical signals. Membrane receptors such as integrins,
20 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 system can sensitively and precisely detect transient changes in morphology and adhesive capacity of the cells, it can be used to monitor cardiac function in vitro. Further, the
25 development of millisecond time resolution permits impedance monitoring of the cardiomyocyte beating cycle as well as fluctuations, even minor alterations, in response to external stimuli.

Exemplary steps involved in using an impedance-measurement system for measurement of cardiomyocyte function include: providing a system for monitoring
30 impedance of excitable cells, in vitro, which is capable of millisecond time resolution, optionally coating wells of the device with either fibronectin or other matrix proteins; seeding either embryonic stem cells (ES cells) of mammalian origin or primary cardiomyocytes at sufficient seeding densities into the wells of the device; allowing

the cells to attach and spread; after a period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

5 The millisecond kinetic readout requires the impedance measurement system be capable of obtaining impedance measurement data at millisecond time resolution. Preferably, the time difference between two consecutive impedance measurements for a well is within the range of milliseconds (e.g., less than 300 millisecond, less than 100 milliseconds, less than 50 milliseconds, less than 20 milliseconds, less than 10
10 milliseconds, or less than or about 1 millisecond). Due to the fast nature of beating cycles of cells, millisecond kinetic readout is required for proper resolution. Thus, the time resolution for the impedance measurement should allow the system to perform measurement using at least two time points for each beat cycle, or at more than two points for each beating cycle. In some embodiments, three, four, five or even ten, or
15 fifteen or twenty or more time points within each beating cycle are obtained.

Embodiments of the present invention include methods of assessing or quantifying cardiomyocytes include providing the system for monitoring impedance of excitable cells in vitro, which includes millisecond time resolution, adding cardiomyocytes to one or more wells, monitoring impedance of the one or more wells,
20 such as using millisecond time resolution, and resolving excitation cycle or beating cycle of the cardiomyocytes. In further embodiments, the method also includes determining cell indices from impedance values, 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. In preferred embodiments, millisecond time
25 resolution permits measurement of at least two consecutive impedance measurements within a beating cycle. In some embodiments at least three consecutive impedance measurements are performed with millisecond time resolution.

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
30 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 system can sensitively and precisely detect transient changes in morphology and adhesive capacity of the cells, the it can be used to monitor cardiomyocyte beating in vitro.

Another exemplary method for impedance based measurement of cardiomyocyte beating includes: providing 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; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding either embryonic stem cells (ES cells) of mammalian origin or primary cardiomyocytes at specific seeding densities to the wells of the device; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

In another exemplary embodiment for measurement of cardiomyocyte function the method includes: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding either ES cells of mammalian origin or primary cardiomyocytes at specific seeding densities; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using an impedance-based system by using millisecond kinetic readout to resolve the individual beat cycles of the cells.

Dynamic monitoring of cardiomyocyte beating is based on quantification in real time of the rhythmic changes in cardiomyocyte morphology as a result of the excitation contraction coupling of the electrically excitable cardiomyocytes growing on microelectrodes' surface of E-PLATES. The quantification of the rhythmic changes in cardiomyocyte morphology is achieved via the fast, millisecond resolution 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. An

exemplary method includes providing a device for measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least one well optionally coated with fibronectin to expedite attachment; adding cells to the at least one well, where the cells can be mouse or human or other
5 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 optionally determining cell indices from impedance values; optionally calculating average rate of beats per unit time, average
10 amplitude intensity in a unit time as well as the average length of time between the beats.

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 β -2 adrenergic receptor agonists can induce morphological changes resulting
15 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 impedance-based system can be used to distinguish between different kinds of morphological effects. The steps involved in using an impedance-
20 monitoring system for measurement of morphological modulation of cardiomyocytes may include: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding 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
25 of the device; allowing the cells to attach and spread; monitoring cardiomyocyte viability over time using the impedance-monitoring system at prespecified intervals of time for 12, 24, 48, 72 hours or longer; at certain times after cell seeding, treat the cell with agents that may cause morphology changes at one or more concentration; using the vehicle that the agent is dissolved in as a control; continuing to monitoring the
30 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; and quantifying 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.

5 Once regular cardiomyocyte beating can be effectively monitored compounds may be added to the wells to assess whether or not beating frequency is altered, beating amplitude is altered and the like. Thus, compounds that adversely affect the normal cardiomyocyte beating are suspected of being cardiotoxic. However, cardioprotective agents can also be assessed by adding an agent suspected of being
10 cardioprotective to the well and inducing a cardiotoxic response.

 Accordingly, methods of identifying or screening for potential agents that modulate ES- derived cardiomyocyte beating or primary cardiomyocyte beating by monitoring and measuring the excitation-contraction coupling of cardiomyocytes upon treatment with pharmacological agents are also provided. The methods may
15 include providing the system for monitoring impedance at millisecond resolution of excitable cells in vitro, adding ES cells or primary cardiomyocytes to two or more wells, adding a test agent suspected of modulating ES-derived or primary cardiomyocyte beating to a first well to provide a test well and either no agent or a control agent to a second well to form a control well, monitoring impedance of the
20 two or more wells at different or similar time intervals using millisecond time resolution at least in part, and optionally determining cell indices from impedance values, generating an impedance-based curve or optionally a cell index curve for each of the test well and control well, and comparing the impedance-based curves or optionally the cell index curves between the test well and the control well. If
25 significantly different, the test agent is concluded to modulate cardiomyocyte function. In preferred embodiments, the impedance-based curves may be direct measurement of cardiomyocyte excitation-contraction coupling; however, the methods may also include long term monitoring of the viability and or morphology of the cardiomyocytes. The methods may show whether the test agent increases
30 cardiomyocyte beating, which is indicative of increased heart rate, or whether the cardiomyocyte beating is slowed, which is indicative of lowered heart rate.

 In another exemplary embodiment a method for screening for modulators of cardiomyocyte beating using an impedance-measurement system in vitro may

include: providing 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; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding 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; allowing the cells to attach and spread; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells; and adding the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency. Preferably, the step of monitoring impedance of beating is performed 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 kinectic readout signals.

In another exemplary embodiment, a method of screening for modulators of cardiomyocyte beating using an impedance-based system in vitro includes seeding cardiomyocytes, in E-PLATES exactly as described in the previous exemplary embodiment; however, prior to addition of pharmacological agent, obtaining a baseline of the cardiomyocyte beating frequency using the impedance measurement system with millisecond time resolution; and adding the pharmacological agents at one or more doses and continue monitoring the cardiomyocyte beating frequency.

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 some embodiments the present invention is directed to a 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 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 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 biologically active agent modulates cardiomyocyte function. Optionally, impedance-based curves or optionally cell index curves are used to calculate the compound dose-dependent changes in cardiomyocyte morphology and generate an EC-50 value for the potency of the compound.

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 cardiomyocytes and cardiomyocyte morphology and differentiation and to screen for pharmacological agents which may modulate these processes and induce cardiotoxicity. In some embodiments, viability is monitored based on long term impedance monitoring of cardiomyocytes seeded in microelectronic plates (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 operably connected to an impedance analyzer, wherein the device includes at least one well optionally coated with fibronectin 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; In another aspect, the method includes providing a device for measuring cell-substrate impedance operably connected to an impedance analyzer, wherein the device includes at least two wells optionally coated with fibronectin 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 of the at least two wells over a period of time and optionally determining cell index from impedance values. 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 or alternatively the cell index of the treated well is significantly different than the impedance or cell index of the treated well.

In other preferred embodiments short term measurement using millisecond time resolution is combined with longer term impedance monitoring, such as over seconds, hours, days and the like. Certain cardiotoxic drugs can directly affect the viability of cardiomyocytes. Thus, long term impedance monitoring may be used to assess loss of viability of cardiomyocytes. An exemplary long term method to assess loss of viability includes: optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding 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 seeding densities to the wells of the device; allowing the cells to attach and spread; monitoring 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; at certain time after cell seeding, treating the cell with a cytotoxic agent at one or more concentration; using the vehicle that the agent is dissolved in as a control; 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, quantify the extent of cardiotoxicity by normalizing the cell index values immediately prior to agent addition and determining 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.

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 morphology operably connected to an optical measurement system, where the device includes at least two wells optionally coated with fibronectin 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 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 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.

One skilled in the art will recognize that any agent may be tested. The agent may be a biologically active agent. Exemplary test agents include compounds, drugs, peptides, proteins, antibodies, antibody fragments, siRNA, shRNA, miRNA, cDNA, lipids and any combination thereof. 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 beats, comparison of these optionally derived parameters is made between the test well and the control well, and if significant differences exist, one may optionally conclude that the test agent modulates cardiomyocyte functions.

The study of cardiomyocyte function and modulation has particular utility in the biomedical arts. For example, the methods of the present invention may be used to assess whether a potential agent is cardiotoxic, cardioprotective and the like. If normal cardiomyocyte beating is altered, the result likely demonstrates the potential

agent is cardiotoxic. Such information would permit effective screening prior to testing in live animals or in humans. Further, by coupling millisecond time resolution over extended time periods, the methods may assess short term and long term effects on cardiomyocytes. Since 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, the systems and methods are able to precisely measure and quantify these changes in cell morphology and shape. Thus the methods and systems have particular utility in the areas of biomedical research.

In some embodiments, potential agents are tested to assess whether they are cardioprotective, or protect against adverse affects. In such embodiments the agent suspected of being cardioprotective is added to the cardiomyocytes prior to, simultaneously or after adding a cardiotoxic agent or a cardiotoxic event. Thus, a cardioprotective agent may prevent abnormal shifts in beating cycle, which would naturally occur in the presence of a cardiotoxic agent. In further embodiments, cardiomyocytes having abnormal beating cycles are treated with potential agents to assess whether normal beating may be induced, such as through administration of a cardioprotective pharmacological agent.

Certain treatments can also affect the differentiative process of ES cells to cardiomyocytes which may involve specific morphological and adhesive changes. In another aspect of the present invention a method is provide to screen or potential agents that may modulate the morphology of ES- derived cardiomyocyte, adult stem cell-derived cardiomyocyte or primary cardiomyocyte or its differentiation.

Impedance-based curves and optionally cell index curves may be used to calculate 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. Significant difference between measurements or data of a test well and a control well support a finding that the biologically active agent within the test well modulates cardiomyocyte function.

E. Assessing the Developmental or Functional Consequence of Gene Knockout and Transgene Expression in Embryonic Stem Cell-Derived Cardiomyocytes

Embodiments of the present invention also include assessing the developmental consequence of genetically manipulated ES cells or their derived cardiomyocytes. 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 with millisecond time resolution can be used to evaluate the role of these genes in cardiac development and or function.

Steps involved in assessing the developmental and functional effect of gene knockout or transgene expression may include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene; provide the system for monitoring impedance of excitable cells having millisecond time resolution; optionally coating wells of the device with either fibronectin or other matrix proteins; seeding the embryonic stem cells (ES cells) of mammalian origin at desired seeding densities into the wells of the device; allowing the cells to attach and spread; after a time period unique to ES-derived cardiomyocytes or primary cardiomyocytes, and monitoring cardiomyocyte beating using the impedance-monitoring system using millisecond kinetic readout to resolve the individual beat cycles of the cells. 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 impedance-based measurement 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. In alternative embodiments, 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 impedance-based measurement system with millisecond time resolution.

In other exemplary embodiments an impedance-based measurement system is used to evaluate the role of 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 may include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene;

providing 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; optionally coat wells of the device with either fibronectin or other matrix proteins; seeding 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; allowing the cells to attach and spread and monitor the growth and viability of the cells using the impedance-based system; after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-monitoring system to monitor electrode impedance by using millisecond kinetic readout to resolve the individual beat cycles of the cells. 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. In related embodiments, 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.

In another exemplary embodiment, steps involved in assessing the developmental and functional effect of gene knockout or transgene expression using an impedance-based system in vitro are provided, which include: obtaining ES cells harboring specific knockout of genes or which expresses a particular transgene; optionally coating E-PLATES with either fibronectin or other matrix proteins; seeding either ES cells of mammalian origin or primary cardiomyocytes at specific seeding densities; allowing the cells to attach and spread; and after a specified period of time unique to ES-derived cardiomyocytes or primary cardiomyocytes, monitoring cardiomyocyte beating using the impedance-based system by using millisecond kinetic readout to resolve the individual beat cycles of the cells. 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 impedance-based 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.

Alternatively, the ES cells can be transfected with specific siRNA to “knockdown” the product of a particular transcript and then monitor cardiomyocyte differentiation
5 and function in vitro using the impedance-based system.

In another aspect, the present invention is directed to method to establish an assay to assess the effect of gene knockout or transgene expression in ES cells differentiated to cardiomyocytes and functionally monitored by the impedance-based system. The method includes providing a device for measuring cell-substrate
10 impedance operably connected to an impedance analyzer, wherein the device includes at least two wells; 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
15 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
20 coupling.

As above, in preferred embodiments, the time difference between two consecutive impedance measurement for a well is in the range of milliseconds (e.g., less than 500 milliseconds, less than 300 milliseconds, less than 100 milliseconds, less than 10 milliseconds, or less than about 1 millisecond or faster). The
25 millisecond 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 using at least two time points for each beat cycle. In some embodiments more than two time points are resolved for each beating cycle.

EXAMPLES

Example 1

Impedance Monitoring of Attachment, Growth and Viability of Mouse ES Cell-Derived Cardiomyocytes

Since isolated primary cardiomyocytes, ES-derived cardiomyocytes and adult stem cell-derived cardiomyocytes can be maintained in culture, these cells provide a desirable model system to study cardiomyocytes as well as cardiomyocyte modulation. The present example demonstrates 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 E-PLATES (Acea Biosciences, San Diego, CA) precoated with fibronectin. The attachment, growth and viability of the cells were monitored on RT-CES system measuring impedance signal in the form of cell index every 30 minute 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.

FIG. 3 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.

Example 2

Detection of Cardiotoxic Effects Using Real Time Impedance Monitoring

As an example, we describe here the use of the ACEA RT-CES system to
5 measure and monitor the attachment and growth of mouse ES cells derived
cardiomyocytes and subsequently treated with a cytotoxic agent (**FIG. 4**).

Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells
per well in E-PLATES precoated with fibronectin. The attachment and growth of cells
were monitored on RT-CES system for 72 hours and then treated with increasing
10 doses of the compound of sodium dichromate dehydrate which is known to induce
cytotoxicity. According to **FIG. 4A** 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
15 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 **FIG. 4B**, half maximal
activity or IC-50 value of 1.48 uM was derived for the compound.

20 Example 3

Real time Monitoring of Mouse ES Cell-Derived Cardiomyocytes and
Inducing Hypertrophy

As an example, we describe here the use of the ACEA RT-CES system to
25 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 (**FIG. 5A**).

Mouse ES-derived cardiomyocytes were seeded at a density of 25,000 cells
per well in E-PLATES precoated with fibronectin. The attachment and growth of the
30 cells were monitored on RT-CES system for 72 hours and then treated with increasing

doses of the compound isoproteranol. According to **FIG. 5A**, 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 (**FIG. 5B**). From the sigmoidal curve generated a half maximal activity or IC-50 value of 3.1 nM was derived for the compound.

Example 4

Real Time Impedance Monitoring with Millisecond Resolution of Beating Cardiomyocytes

As an example, we describe here the use of an improved impedance-based system with millisecond time resolution to measure and monitor the beating of cardiomyocytes using fast kinetic software.

Mouse ES cells were seeded at a density of between 3,000 to 50,000 cells per well in E-PLATES precoated with fibronectin. The attachment and growth of the cells were monitored on RT-CES system. **FIG. 6** 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 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 up to 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. We now understand the origins of such “small-spikes” and interpret that such spikes in the impedance or cell index readout are associated with the 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 milliseconds) 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 **FIGS. 3** and **6**, where the background measurement is performed using cell culture media). The software was used to measure cardiomyocyte beating at distinct stages throughout its attachment and growth phases (**FIG. 7**). 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 (**FIG. 7A**). For plot, impedance readout has been converted into dimensionless cell indices. **FIGS. 7B** and **7C** 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.

- 5 The algorithm needs 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 cardiomyocytes 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
10 method of signal 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,
15 the beating frequency. 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. **FIG. 8A** and **FIG. 8B** shows a pair of cell index curves and the corresponding beat rate based the above described Fourier transform. In **FIG. 8B**, the cell index curves last from time zero to time 42 seconds. In **FIG. 8B**,
20 the beating frequency for the traces in **FIG. 8A** 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 **FIG. 8B**, 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 **FIG. 8B**
25 starts 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 two-consecutive peaks, one can calculate one beating rate.
30 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. **FIG. 9A** 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 uM. Corresponding cell index data is shown in **FIGS. 9C** and **9D**, where the time resolution between two adjacent points is 40 milliseconds. In other words, a second in **FIGS. 9C** and **9D** is equivalent to 40-milliseconds.

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. **FIG. 9B** 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 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 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.

Example 5

Identifying Modulators of Cardiac Function Using Real Time Impedance Monitoring with Millisecond Resolution

Similar to Example 4, the millisecond kinetic readout requires that the impedance measurement system can provide impedance measurement data at millisecond time resolution. In other words, the time difference between two consecutive impedance measurement for a well shall be in the range of milliseconds (e.g., less than 500 millisecond, less than 300 millisecond, less than 100 millisecond, less than 10 millisecond, or less than 1 millisecond or faster). The millisecond

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.

5 In order to demonstrate the utility of the millisecond kinetic impedance-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 differentiated into beating cardiomyocytes, as described in Section 4. 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 final concentration of 333 nM and cardiomyocyte beating was monitored for 10 minutes (**FIG. 10A**). 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 isoproteranol at a final concentration of 4.4 uM significantly increased the rate of cardiomyocyte beating from ~ 65 beats/min to 115-135 beats/min (**FIG. 10B** 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 **FIGS. 9C** and **9D**, the time resolution between two adjacent points in **FIGS. 10A** and **10B** is 40 milliseconds. In other words, a second in **FIGS. 10A** and **10B** is equivalent to 40 millisecond.

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

To further demonstrate the capabilities of the improved 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 effects 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 RT-CES system. Approximately, 72 hours after cell seeding the improved, impedance-measurement system with millisecond 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 the indicated drug and dose shown in **FIG. 11**. Similar to the cell index plot shown in **FIGS. 9C** and **9D**, the time resolution between two adjacent points in all the figures in **FIG. 9** is 40 milliseconds. In other words, a second in **FIG. 9** is equivalent to 40-milliseconds.

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 **FIG. 12** some of these compounds can lead to qualitatively different or similar patterns. For example, the compounds E4031, Astezimole and dofetilide which are ERG channel inhibitors do contain patterns with similar features. In summary these results clearly indicate that using the impedance-measurement system with millisecond time resolution 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 **FIGS. 9C** and **9D**, the time resolution between two adjacent points in all the figures in **FIG. 10** is 40 milliseconds. In other words, a second in a figure in **FIG. 10** is equivalent to 40-millisecond.

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 μ M	From ~ 60 to ~ 80	0.09 to 0.07	No pattern change
Moxifloxacin	anti-biotic	20 μ M	From ~ 80 to ~ 73	From 0.055 to 0.060	No pattern change
Pentamidine	anti-infective	20 μ M	From 71~78 to ~71	From 0.24 to 0.21	No pattern change
Amitriptyline	Serotonergic Inhibitor	4.4 μ M	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 μ M	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 μ M	From ~ 68 to ~ 60	From 0.2 to 0.19	No pattern change
Rofecoxib	COX-2 Inhibitor	40 μ M	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 μ M	From ~ 60 to (~20 ~ 50)	From ~0.2 to ~0.12	Pattern change
Doxirubicin	Anthracycline	40 μ M	~ 70	~ 0.16	No pattern change (initially). Beating pattern changes after 2 hrs.
Cyclosporin A	Calcineurin inhibitor	13.3 μ M	From ~70 to ~ 80	From 0.18 to 0.15	No pattern change
Propalanol	β 2-adrenergic receptor antagonist	4.4 μ M	From ~70 to over 150	From 0.25 to 0.025	Pattern changed, much faster and irregular
Sotalol	K channel inhibitor	13.3 μ M	From ~ 80 to ~ 160	From 0.27 to 0.07	Much faster, pattern changed
E4031		120 nM	From 80 to 160 From 80 to 140, then to ~ 80	From 0.09 to 0.03	Pattern changed, much faster
DDT	Pesticide	8 μ M	From ~ 65 to 0	From 0.25 to 0.02	Initially become faster, later irregular beating
PCB	Organic toxicant	8 μ M	From ~ 75 to 0	From 0.2 to 0	Beating stopped
Endosulfan	insecticide	8 μ M	From ~ 75 to 0	From 0.2 to 0	Beating stopped

To further demonstrate the capabilities of the fast impedance-measurement-system-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.

5 Approximately, 72 hours after cell seeding the impedance-measurement system with millisecond 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. **FIG. 13** shows an example of dose dependent effects of Astemizole on cardiomyocytes beating at different
10 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
15 in **FIGS. 9C** and **9D**, the time resolution between two adjacent points in all the figures in **FIG. 11** is 40 milliseconds. In other words, a second in **FIG. 13** is equivalent to 40 milliseconds.

Example 6

20 Modulation of Cardiomyocyte Beating In Response to Haloperidol Treatment Using Millisecond Time Resolution

To further demonstrate the capabilities of the improved impedance measurement system for monitoring of cardiomyocyte beating in detecting drugs
25 which may adversely affect heart function, 500nM Haloperidol was tested to demonstrate its time-dependent effects on cardiomyocytes. Approximately, 72 hours after cell seeding the improved impedance system was used to establish a baseline reading of cardiomyocyte beating for each well for about 16 seconds (**FIG. 14A**). Note that in **FIG. 14A**, each minute showed on the x-axis of **FIG. 14A** corresponds to
30 one second in real measurement. Subsequently, the cells in the well treated with 500 nM Haloperidol. **FIGS. 14B, C, D, E, F, G** and **H** show the impedance-based monitoring of beating of the cardiomyocytes at time points of first 30 seconds, about

1 minute, about 2 minutes, about 4 minutes, about 5.5 minutes, about 1 hour and about 21 hours after the compound treatment. In **FIGS. 14B** through **14H**, each minute showed on the x-axis corresponds to one second in real measurement. It is clear that within 1 hr of compound treatment, the cardiomyocytes exhibit significant, time-dependent change in their impedance responses. Even at ~ 21 hrs after compound treatment, the impedance pattern did not restore and the beating frequency (50 ~ 60 beats per minute) appeared to become about half of that before compound treatment (90 ~ 100 beats per minute before treatment). Yet the amplitude of the impedance response spikes nearly doubled compared with that before compound treatment. **FIG. 15** and **FIG. 16** show the analysis results of the impedance beating pattern of **FIGS. 14** in terms of the beating frequency (beats per minute) and the beating magnitude. For **FIG. 15** and **FIG. 16**, each unit (1) on the x-axis corresponds to 0.016 second (16 milliseconds) in real measurement.

Example 7

Modulation of Cardiomyocyte Beating In Response to Eryromycin Treatment Using Millisecond Time Resolution

To further demonstrate the capabilities of the improved impedance measurement system for monitoring of cardiomyocyte beating in detecting drugs which may adversely affect heart function, 300 uM erythromycin was tested to demonstrate its time-dependent effects on cardiomyocytes. Approximately, 72 hours after cell seeding the improved impedance system was used to establish a baseline reading of cardiomyocyte beating for each well for about 15 seconds (**FIG. 17A**). Note that in **FIG. 17A**, each minute showed on the x-axis of **FIG. 17A** corresponds to one second in real measurement. Subsequently, the cells in the well treated with 300 uM erythromycin. **FIGS. 17B-I** show the impedance-based monitoring of beating of the cardiomyocytes at time points of first 30 seconds, about 1 minute, about 2 minutes, about 4 minutes, about 5.5 minutes, about 1 hour, about 3hr and about 21 hours after the compound treatment. In **FIGS. 17B-H**, each minute showed on the x-axis corresponds to one second in real measurement. It is clear that within 3 hr of compound treatment, the cardiomyocytes exhibit significant, time-dependent change

in their impedance responses. For example, at about 1 hr after the treatment, the frequency of the impedance-based beating spikes is much higher than that for the cardiomyocytes before treatment, yet the magnitude is much smaller. On the other hand, at about 3 hr after treatment, the impedance-based beating pattern becomes
5 rather irregular whilst the magnitude of the beating pattern has somewhat restored. Still, even at ~ 21 hrs after compound treatment, the impedance pattern did not restore and the beating frequency appeared to about 70% (at 50 ~ 60 beats per minute) of that before compound treatment (at 80 ~ 90 beats per minute). Yet the amplitude of the impedance response spikes increased by about 50% compared with that before
10 compound treatment. **FIG. 18** and **FIG. 19** show the analysis results of the impedance beating pattern of **FIGS. 17** in terms of the beating frequency (beats per minute) and the beating magnitude. For **FIG. 18** and **FIG. 19**, each unit (1) on the x-axis corresponds to 0.016 second (16 milli-seconds) in real measurement.

CLAIMS

What is claimed is:

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1. A system for monitoring impedance of excitable cells in vitro, comprising:

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a) a device for monitoring cell-substrate impedance, comprising

i) a nonconductive substrate;

ii) one or more electrode arrays fabricated on said substrate;

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iii) one or more wells on said substrate, wherein each of said one or more arrays is associated with one of said one or more wells; wherein each electrode array is individually addressable, further wherein said substrate has a surface suitable for cell attachment, wherein said cell attachment on said substrate can result in a detectable change in impedance between electrodes within each electrode array;

b) an impedance analyzer capable of impedance measurement at millisecond time resolution;

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c) electronic circuitry that can engage said device and selectively connect said two or more electrode arrays of said device to said impedance analyzer; and

d) a software program that controls said electronic circuitry and records and analyzes data obtained from said impedance analyzer.

25

2. The system according to claim 1, wherein each of said electrode arrays comprises two electrode structures having substantially a same surface area.

30

3. The system according to claim 2, wherein said electrode structures are each constructed from a material selected from the group consisting of metal, gold, platinum, titanium and chromium.

4. The system according to claim 2, wherein said electrode structures have a width between 20 microns and 500 microns.

5. The system according to claim 2, wherein a gap between electrode
5 structures within each electrode array is between 5 microns and 100 microns.

6. The system according to claim 1, wherein each electrode array is organized in a configuration selected from the group consisting of interdigitated, circle-on-line, diamond-on-line, concentric, sinusoidal, and castellated.
10

7. The system according to claim 1, wherein said millisecond time resolution allows for impedance measurements at more than two time points within each excitation cycle of excitable cells introduced to wells.

8. The system according to claim 7, wherein said excitation cycle is a cell
15 beating cell.

9. The system according to claim 8, wherein said excitable cells are cardiomyocytes.
20

10. The system according to claim 7, wherein said excitable cells are excitation-contraction coupling cells.

11. The system according to claim 1, wherein said impedance
25 measurement at millisecond time resolution comprises at least two consecutive impedance measurements are less than 100 milliseconds apart.

12. The system according to claim 11, wherein said at least two consecutive impedance measurements are less than 40 milliseconds apart.
30

13. The system according to claim 12, wherein said at least two consecutive impedance measurements are less than 20 milliseconds apart.

14. The system according to claim 13, wherein said at least two consecutive impedance measurements are less than 10 milliseconds apart.

15. The system according to claim 1, wherein said one or more wells is
5 selected from a group of 6 wells, 8 wells, 16 wells, 32 wells, 96 wells and 384 wells.

16. A method for assessing and quantifying excitable cells in vitro, comprising

- 10 a) providing the system according to Claim 1;
 b) adding excitable cells to said one or more wells;
 c) monitoring impedance of said one or more wells using
 millisecond time resolution; and
 d) resolving individual cycles of said excitable cells.

15 17. The method according to claim 16, wherein said excitable cells are cells undergo excitation-contraction coupling.

 18. The method according to claim 17, wherein said excitable cells are cardiomyocytes.

20

 19. The method according to claim 16, wherein the step of monitoring impedance comprises performing impedance measurements at millisecond time resolution simultaneously across all wells.

25 20. The method according to claim 19, wherein said impedance measurements at millisecond time resolution comprises at least two consecutive impedance measurements at less than 300 milliseconds apart.

 21. The method according to claim 20, wherein said at least two
30 consecutive impedance measurements are less than 100 milliseconds apart.

 22. The method according to claim 21, wherein said at least two consecutive impedance measurements are less than 40 milliseconds apart

23. The method according to claim 22, wherein said at least two consecutive impedance measurements are less than 10 milliseconds apart.

24. A method of determining whether a compound modulates a beating cycle of an excitable cell, comprising:

a) providing the system according to claim 1, wherein said system comprises at least two wells;

b) adding excitable cells to said at least two wells;

c) monitoring impedance of said at least two wells using millisecond time resolution;

d) adding a compound suspected of modulation of the beating cycle to a first of said at least two wells to provide a test well, wherein a second well lacking compound is provided as a control well;

e) resolving the beating cycles of both test well and control well; and

d) comparing said beating cycles between said test well and control well, wherein a difference in beating cycles indicates said compound modulates the beating cycle of the excitable cell.

25. The method according to claim 24, wherein the excitable cells are cardiomyocytes or embryonic stem cell derived cardiomyocytes.

26. The method according to claim 24, wherein said beating cycles are compared to identify changes in beating amplitude.

27. The method according to claim 24, wherein said beating cycles are compared to identify changes in beating frequency.

28. The method according to claim 27, wherein said changes in beating frequency are regular changes or irregular changes.

29. The method according to claim 24, further comprising determining a cell index for each of said test well and control well, wherein the step of comparing

said beating cycles comprises comparing said cell indexes between test well and control well.

30. A method of characterizing rhythmic beating of a cardiomyocyte
5 comprising:

- a) providing the system according to claim 1;
- b) adding excitable cells to said one or more wells;
- c) monitoring impedance of said one or more wells in millisecond
time resolution; and
- 10 d) determining a plurality of beating cycle peaks in a time unit;
and
- e) comparing amplitude or frequency of said peaks over said time
unit.

15 31. The method according to claim 30, wherein the step of determining a plurality of beating cycle peaks comprises determining a rise in impedance and a decay in impedance and extrapolating a peak from each rise and decay.

20 32. The method according to claim 30, further comprising determining a cell index for each of said one or more wells, wherein the step of determining a plurality of beating cycle peaks comprises for each cycle determining a rise in cell index, a decay in cell index and extrapolating each peak from each rise and decay.

25 33. The method according to claim 30, wherein beating cycle peaks are determined by identifying impedance data points at which the first order derivative to time is zero or close to zero.

30 34. The method according to claim 30, further comprising monitoring impedance of said one or more wells over seconds, minutes or hours, optionally wherein said monitoring of impedance in millisecond time resolution is performed within said monitoring over seconds, minutes or hours.

35. A method of assessing genetic manipulation of an embryonic stem cell, comprising:

a) providing the system according to claim 1, wherein said device comprises at least two wells;

5 b) mutating embryonic stem cells to comprise a genetic mutation;

c) adding said mutated embryonic stem cells to a first well of said at least two wells to form a test well and control embryonic stem cells to a second of said at least two wells to form a control well;

10 c) monitoring impedance of said at least two wells in millisecond time resolution; and

e) resolving the beating cycles of both test well and control well, if any; and

d) comparing said beating cycles between said test well and control well, wherein a difference in beating cycles indicates said genetic mutation modulates embryonic stem cell differentiation.

36. The method according to claim 35, wherein said embryonic stem cell differentiation is differentiation into a cardiomyocyte.

20 37. The method according to claim 35, wherein said genetic mutation is selected from the group consisting of a genetic knock out, a knock down and an inserted transgene.

25 38. A method for assessing and quantifying excitable cells in vitro, comprising

a) providing the system according to claim 1;

b) adding excitable cells to said one or more wells;

30 c) monitoring impedance of said one or more wells over a first time period, which is characterized as a longer time period comprising seconds, minutes or hours;

d) monitoring impedance of said one or more wells over a second time period in millisecond time resolution;

e) determining a characteristic selected from the group consisting of cell attachment, cell growth and cell viability from monitoring over said first time period; and

f) resolving individual cycles of said excitable cells from monitoring over said second time period.

39. The method according to claim 38, wherein monitoring impedance over said second time period is performed at regular or irregular time intervals within monitoring impedance over said first time period.