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(71) Applicant: **STEMONIX INC.** [US/US]; 9081 Waverly Ct, Eden Prairie, Minnesota 55347 (US).

(72) Inventor; and

(71) Applicant: **CARROMEU, Cassiano** [BR/US]; 8840 Costa Verde Blvd, Apt 3427, San Diego, California 92122 (US).

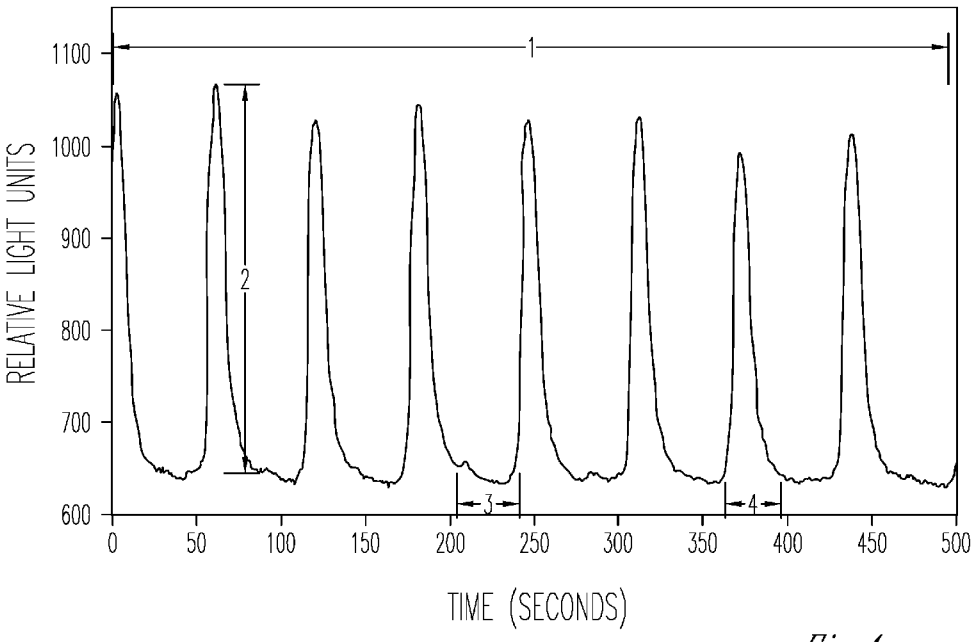
(74) Agent: **BIANCHI, Timothy, E.** et al.; Schwegman Lundberg & Woessner, P.A., P.O. Box 2938, Minneapolis, Minnesota 55402 (US).

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(54) **Title:** HIGH THROUGHPUT OPTICAL ASSAY OF HUMAN MIXED CELL POPULATION SPHEROIDS



GRAPH SHOWING 8 PEAKS 30

Fig. 4

(57) **Abstract:** The present disclosure provides a method of performing a functional assay on human spheroids, e.g., three-dimensional human cell spheroids using, in one embodiment, a fluorometric imaging plate reader.

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HIGH THROUGHPUT OPTICAL ASSAY OF HUMAN MIXED CELL POPULATION SPHEROIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. application Serial No. 62/532,667, filed on July 14, 2017, the disclosure of which is incorporated by reference herein.

BACKGROUND

The accelerating pace of drug discovery has spawned an increasing need for functional *in vitro* assays using living human cells. Automating these assays for high-throughput systems, however, has proven to be difficult. Noting that the most common biological assays employed in high-throughput settings rely on some kind of fluorescent measurement.

Fluorometric Imaging Plate Reader, otherwise known as the FLIPR, is a unique combination of optics, automated pipetting, and temperature control. Designed to perform high-throughput screening assays using adherent and nonadherent cells, FLIPR is ideally suited for kinetic, cell-based assays such as measuring changes in intracellular calcium levels and membrane potential.

FLIPR integrates a powerful argon laser, a CCD (charged couple device) imaging camera, an optical detection scheme, and a programmable 96-well pipettor to perform fluorometric analyses on all 96 wells of a microplate simultaneously. The system generates real-time kinetic data by stimulating and reading all 96 wells in 1-second intervals. Typically, the argon laser excites a suitable fluorescent dye. The resultant emitted light is detected by a cooled CCD camera that acts as an integrated detector, accumulating signal over the duration of the exposure. Sensitivity is further enhanced by the FLIPR's optical scheme, which limits the depth of field of the CCD camera to a few hundred microns on the bottom of each well, or essentially at the level of the cell monolayer. This technique reduces the background fluorescence from extracellular dye by about an order of magnitude. Taken together, these systems combine into an extremely sensitive fluorescence detector.

The FLIPR instruments capture real-time kinetic data, enabling the identification of a potential drug hit within seconds of its addition to an assay.

Indeed, one of the features of FLIPR technology is its data fidelity, allowing researchers to monitor single-well determinations. The software also performs data analysis and reduction and can export data to spreadsheet programs.

The use of FLIPR with 2 dimensional cell formats in which the cells of interest are added to a microplate well and form a monolayer across the well bottom has proven to be problematic because the cells do not always fill the wells uniformly or attached with high fidelity. This leads to inconsistent data generation measured by FLIPR within a plate and also from plate to plate. When conducting a high throughout screen on 96, 384 or 1536 well plates, data consistency is needed to generate meaningful information on drug interaction or drug kinetic data.

SUMMARY

Surprisingly, the modulation of calcium oscillations by drug molecules on a 3D spheroid can be monitored and the resulting data is quite consistent, e.g., spheroid neuron synapses fired predictably and consistently for long periods of time. In one embodiment, the disclosure provides an optical assay, e.g., a functional FLIPR assay or high content high magnification optical microscopy, of 3D human cell spheroids e.g., mixed population human cell neuron spheroids. In one embodiment, prior to testing, spheroids are cultured for 4 to 16 weeks to induce robust synchronized synaptic networks to mimic mature human like brain functionality. Those mixed population spheroids fired predictably and consistently for long periods of time. In one embodiment, the disclosure provides a high throughput optical assay of a mixed population of human cell 3D spheroids utilizing FLIPR and calcium uptake fluorescence oscillations. The oscillations could be modulated with chemical compounds, and oscillatory firing can be altered with agonist or antagonists.

In one embodiment, the disclosure provides an optical method to detect the effect of one or more compounds on spheroids. The method includes contacting a tissue culture plate, e.g., a plate having wells, comprising one or more spheroids of human cells such as those of uniform diameter and one or more test compounds; and optically detecting the amount or change in oscillations in the spheroids. In one embodiment, the plate is a multi-well plate. In one embodiment, the spheroids are further contacted with a fluorescent

molecule useful to detect calcium, and the amount or change in fluorescence over time is detected. In one embodiment, the amount or change in fluorescence is detected via a quantity of peaks of fluorescence, an amplitude of one or more of the peaks, peak spacing between one or more of the peaks, a width of one or more peaks, or any combination thereof. In one embodiment, the spheroids comprise neurons. In one embodiment, the spheroids comprise neurons and astrocytes. In one embodiment, the spheroids comprise heart, liver, kidney, pancreas, lung, endothelial or epithelial cells. In one embodiment, the spheroids comprise cancer cells or immortalized cells. In one embodiment, the spheroids comprise microglial cells or oligodendrocytes. In one embodiment, the spheroids comprise pericytes and endothelial cells. In one embodiment, the spheroids comprise endothelial cells, microglial cells, neurons, oligodendrocytic cells, or any combination thereof. In one embodiment, the cells are differentiated cells. In one embodiment, wherein the cells are progenitor cells such as human iPSCs. In one embodiment, the progenitor cells are progenitors of neurons, astrocytes, heart cells, liver cells, kidney cells, pancreas cells, lung cells, endothelial cells, or epithelial cells. In one embodiment, the one or more spheroids have a diameter of about 500 to about 600 microns. In one embodiment, the one or more spheroids have a diameter of about 450 to about 500 microns. In one embodiment, the one or more spheroids are cultured for at least 4 to 6 weeks before contacting with the one or more test compounds. In one embodiment, the fluorescent molecule comprises Calcium 3, Calcium 4, Calcium 5, Calcium 6, Fluo 3, or Fluo4, or a combination thereof. In one embodiment, the one or more spheroids are further contacted with a cell membrane impermeant quencher. In one embodiment, the amount of change in fluorescence is compared to the fluorescence with one or more spheroids and the fluorescent molecule but no test compound. In one embodiment, in a multi-well plate each well has one spheroid.

Also provided is a multi-well plate comprising one or more mixed human cell spheroids per well. In one embodiment, the spheroids comprise neurons and astrocytes. In one embodiment, the spheroids comprise heart, liver, kidney, pancreas, lung, endothelial or epithelial cells. In one embodiment, the spheroids comprise microglial cells or oligodendrocytes. In one embodiment, the

spheroids comprise pericytes and endothelial cells. In one embodiment, the spheroids comprise endothelial cells, microglial cells, neurons, oligodendrocytic cells, or any combination thereof. In one embodiment, the spheroids comprise progenitors of neurons, astrocytes, heart cells, liver cells, kidney cells, pancreas cells, lung cells, endothelial cells, or epithelial cells.

These and other objects and advantages of the invention will become apparent from reading and understanding the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is an image of an exemplary 384 well microplate with 1 mixed cell spheroid located in each well, to demonstrate one embodiment of the present subject matter.

Fig. 2 is a plot of average mixed cell spheroid size across a number of columns in a 384 well microplate in one example of an application of the present subject matter.

Fig. 3 is an image of a mixed population neuron spheroid.

Fig. 4 is a FLIPR control plot of fluorescence generated by a spheroid from a micro plate well.

Fig. 5 is a FLIPR data plot of a spheroid control well vs. drug challenged well, demonstrating variation of response for a drug challenged well versus a control well, to demonstrate one application of the present subject matter.

Fig. 6 is a summary of various Glutametergic and Gabaergic antagonists and agonist effects on spheroids as measured by FLIPR, in one example application of the present subject matter.

DETAILED DESCRIPTION

The following discussion is directed towards various embodiments of the invention. Although one or more of these embodiments may be preferred, the invention is not limited to the embodiments disclosed. In addition, one skilled in the art will understand that the following description has broad application, and the discussion of any embodiment is meant only to be exemplary of that embodiment, and not intended to limit the scope of the disclosure or claims to that embodiment.

Fig. 1 is an image of an exemplary 384 well microplate with 1 mixed cell

spheroid located in each well, to demonstrate one embodiment of the present subject matter. In this example, 24 columns of 16 cells of the microplate provide the 384 wells. Fig. 1 shows an actual black and white image of an entire 384 well microplate **10** into which each well is formed a spheroid **20** having a mixed population of astrocytes and neurons that forms a structure that is approximately 500 microns in diameter. The microplate can have, in one embodiment, 96, 384 or 1536 wells. Other numbers of wells are possible without departing from the scope of the present subject matter. The spheroids **20** may be formed by adding individual cells into each well that subsequently spontaneously form into different size spheroids depending on the number of cells that are initially added. The number of cells inserted into each well can range from hundreds to hundreds of thousands depending on well size and the diameter of the spheroid desired for the assay. In one embodiment, approximately 20,000 human iPS cells are added to the well and form a spheroid approximately 500 microns in diameter.

In Fig. 1, a microBrain ™ 3D plate is used to demonstrate the microplate; however, it is understood that other microplates and structures may be used to create a plurality of wells.

Fig. 2 shows a plot of spheroid **20** diameters (i.e., "width") vs. location across the 24 columns of a 384 well microwell plate. As can be seen from the data the diameter uniformity and size is very consistent. The ratio of the astrocyte to neurons in the mixed population can range from about 5 to about 95 percent, about 10 to about 90 percent, about 20 to about 80 percent, about 40 to about 60 percent, about 60 to about 40 percent, about 80 to about 20 percent, about 90 to about 10 percent, or about 95 to about 5 percent, e.g., a ratio of 50/50 may mimic what is found in the human brain. The cells used to form the spheroids can be derived from human primary cell lines, human iPScs or human engineered immortal cell lines. Also the spheroids are not limited to only neurons but can also be heart, liver, kidney, pancreas, lung, endothelial and epithelial cells as well as solid tumor cell lines. Other cells may be used without departing from the scope of the present subject matter.

Fig. 3 shows a black and white image of an actual mixed population neuron spheroid **20** from an individual well within a microwell plate. What is unique about neuron spheroids is that they self organize into very uniform round geometries. The diameter of the spheroids can range from about 100 microns to

about 10 millimeters, e.g., about 200 to 600 microns in diameter, e.g., about 300 to about 500 microns in diameter, about 400 to about 600 microns in diameter, or about 450 to about 650 microns in diameter, or about 475 to about 525 in diameter, e.g., an average of about 500 microns in diameter. In this particular example the ratio of astrocytes to neurons is approximately 50/50.

Referring to Fig 4, this plot show the type of data generated by a FLIPR assay on a mixed population neuron spheroid **20** as a function of time. The parameters that are measured are peak count 1, peak amplitude 2, peak spacing 3, and peak width 4. All of these variables may be employed when interpreting the data generated by a FLIPR assay. They are indirect measurements of how the cells are behaving at steady state (no drug challenge) and how drugs are interacting with the cells in the spheroid **20**. The peaks **30** represent spontaneous fluorescing calcium oscillations being generated by the spheroid in culture media. Data for each cell can be obtained and stored using FLIPR.

Fig. 5 shows the effect of the drug Bicuculline that is a GABAergic antagonist at a 50 micro molar concentration on inducing an irregular and erratic calcium uptake behavior in the spheroids **20** vs. the controls. The axes of the figure show "Relative Light Units" and "Time (Seconds)." A comparison of the Bicuculline drug peaks to those of the control shows almost all of the measurement parameters are altered such as peak number, spacing and height. The same type of response was also observed using Glutametergic antagonist as well but at higher dose concentrations. It should be noted that the age of the spheroids may have an effect on the drug interactions. In the one embodiment, the spheroids can range in age from 2 to over 52 weeks old in order to simulate more mature cells as found in *in vivo* models. In one embodiment, 5 to 10 week old spheroids **20** are employed. For example, aged spheroids formed of neurons, e.g., aged about 8 weeks, allow for more synapse connections.

Fig. 6 is a summary of some of the results obtained using the FLIPR assay and spheroids. The graph on the left shows the change in the number of calcium oscillation peaks generated before (control) and after exposure to well known Glutametergic and Gabaergic agonists and antagonists small molecule drug compounds used from the table on the right. As can be seen from the graph on the right there is a significant and immediate modulation of the spheroid **20** calcium oscillations with the exposure to the listed drug compounds. This type

of chemically induced response can be the basis for an epilepsy seizure model that mimics what is observed in humans. Therefore, this 3D spheroid model for example can be used as a high throughput-screening tool for drugs that can rescue erratic neuron synapse firing that is the basis for seizures and other diseases.

Thus, in one embodiment, the present disclosure provides a method of performing a functional assay on a mixed population of three-dimensional human cell spheroids using, in one embodiment, a fluorometric imaging plate reader. For example, very uniform and consistent mixed population spheroids, e.g., astrocyte and neuron spheroids, may be generated from differentiated cell such as human iPS cells. The spheroids may range in diameter from, in one embodiment, about 500 to about 600 microns, and may be formed in wells of a multi-well plate, e.g., formed in a 384 well micro plate. The spheroids are then contacted with one or more molecules, e.g., Gabaergic and Glutametergic modulating small molecules. The temporal response of calcium oscillation fluorescent light intensity of the spheroids may be captured and quantified in real time and is a measure of the cell spheroid response to drug challenges and concentration gradients.

Some Examples of the Present Subject Matter

Spheroids, such as those formed from two or more different cell types, may be prepared using any suitable medium, optionally including one or more different growth factors, and any suitable conditions. For example, spheroids formed from neurons and astrocytes may be prepared using, in one embodiment, one or more of the following media and/or conditions: BrainPhys™ Neuronal Medium (StemCell Tech) supplemented 1x with SM1 Neuronal Supplement (BrainPhys™ Neuronal Medium and SM1 Kit (cat. # 05792; StemCell Technologies), 20 ng/mL BDNF (cat. # 78005; StemCell Technologies), 20 ng/mL GDNF (cat. # 78058; StemCell Technologies) and penicillin/streptomycin (cat. # SV30010; GE Healthcare Life Sciences). The cells are maintained at 37°C in an incubator with 5% CO_2 and high humidity.

The present subject matter allows for multiple approaches for analyzing the effects of one or more compounds on spheroids, comprising contacting a plate, e.g., a multi-well plate having wells, comprising one or more spheroids of

human cells of uniform diameter, a fluorescent molecule useful to detect calcium, and one or more test compounds; and optically detecting the amount or change in fluorescence over time, e.g., in each well. In various examples, the method detects the amount or change in fluorescence via a quantity of peaks of fluorescence, an amplitude of one or more of the peaks, peak spacing between one or more of the peaks, a width of one or more peaks, or any combination thereof. In various examples, the foregoing methods may include wherein the spheroids comprise neurons or wherein the spheroids comprise neurons and astrocytes, or wherein the spheroids comprise heart, liver, kidney, pancreas, lung, endothelial or epithelial cells, or wherein the spheroids comprise cancer cells. In various of the preceding examples, the spheroids may comprise a plurality of different cell types. In the foregoing examples, some examples include wherein the cells are derived from human iPSCs. In some examples, the cells are differentiated cells. In some examples, the cells are progenitor cells. In some examples using progenitor cells, the progenitor cells are progenitors of neurons, astrocytes, heart cells, liver cells, kidney cells, pancreas cells, lung cells, endothelial cells, or epithelial cells. In some of the foregoing examples, the cells are immortalized cells.

In various of the foregoing methods, the spheroids may have a diameter of about 500 to about 600 microns or a diameter of about 450 to about 500 microns. In various of the foregoing methods, the spheroids may have been in culture for at least 6 weeks. In various of the foregoing methods, including a fluorescent molecule, the molecule comprises Calcium 3, Calcium 4, Calcium 5, Calcium 6, Fluo 3, or Fluo4.

In one embodiment, an optical assay is provided, e.g., a functional FLIPR assay or high content high magnification optical microscopy, of 3D human cell spheroids, e.g., spheroids formed of mixed populations of neurons, oligodendrocytes, microglial cells, endothelial cells, or any combination thereof.

In one embodiment, a multi-well optical assay is provided, such as a functional FLIPR assay, of 3D mixed population human cell spheroids in a multi-well format, e.g., a 96, 384 or 1536 microplate well, e.g., spheroids in a rounded bottom well format.

Further provided is an optical assay, e.g., a functional FLIPR assay, of 3D mixed population spheroids in which the spheroids in each microplate well

are of uniform size, e.g., diameters that are +/- 50 or +/- 25 microns. In one embodiment, FLIPR generates real time functional data on 3D neuron based cell spheroids that are very consistent within a micro plate, e.g., well-to-well, and from plate to plate.

In one embodiment, the disclosure provides an optical assay including a functional FLIPR assay of 3D mixed population spheroids that respond to agonist or antagonist drug challenge in real time.

In one embodiment, the disclosure provides an optical assay, e.g., a functional FLIPR assay, of 3D mixed population spheroids derived from human primary cells, iPSc, differentiated cells, or various immortal human cell lines.

In various of the foregoing methods, further comprising contacting the wells with a cell membrane impermeant quencher. In any of the foregoing methods the amount of change in fluorescence can be compared to the fluorescence in a well with spheroids and the fluorescent molecule but no test compound.

Those skilled in the art will understand other examples and variations are possible without departing from the scope of the present subject matter.

The above discussion is meant to be illustrative of the principle and various embodiments of the present invention. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention Thus, numerous variations and modifications will become apparent to those skilled in the art once the above disclosure is fully appreciated. For example the invention is not limited to neurons or mixed populations of neurons. The invention can be applied to all organ types found in humans such as heart, lung, liver, kidney, colon, pancreas and cancer mixed population cell spheroids to name a few. It is intended that the following claims be interpreted to embrace all such variations and modifications.

WHAT IS CLAIMED IS:

1. An optical method to detect the effect of one or more compounds on spheroids, comprising:
contacting one or more spheroids of human cells of uniform diameter and one or more test compounds; and
optically detecting the amount or change in oscillations of one or more of the spheroids.

2. The method of claim 1 wherein the one or more spheroids are in wells of a multi-well plate.

3. The method of claim 2 wherein each well has one spheroid.

4. The method of claims 2 or 3 wherein the wells are further contacted with a fluorescent molecule useful to detect calcium, and the amount or change in fluorescence over time is detected in one or more wells.

5. The method of claim 4 wherein the amount or change in fluorescence is detecting a quantity of peaks of fluorescence, an amplitude of one or more of the peaks, peak spacing between one or more of the peaks, a width of one or more peaks, or any combination thereof.

6. The method of any one of claims 1 to 5 wherein the one or more spheroids comprise neurons.

7. The method of any one of claims 1 to 5 wherein the one or more spheroids comprise neurons and astrocytes.

8. The method of any one of claims 1 to 7 wherein the one or more spheroids comprise heart, liver, kidney, pancreas, lung, endothelial or epithelial cells.

9. The method of any one of claims 1 to 8 wherein the one or more spheroids comprise cancer cells or immortalized cells.

10. The method of any one of claims 1 to 9 wherein the one or more spheroids comprise microglial cells or oligodendrocytes.

11. The method of any one of claims 1 to 10 wherein the one or more spheroids comprise pericytes and endothelial cells.

12. The method of any one of claims 1 to 11 wherein the one or more spheroids comprise endothelial cells, microglial cells, neurons, oligodendrocytic cells, or any combination thereof.

13. The method of any one of claims 1 to 12 wherein the cells are progenitor cells.

14. The method of claim 13 wherein the progenitor cells are progenitors of neurons, astrocytes, heart cells, liver cells, kidney cells, pancreas cells, lung cells, endothelial cells, or epithelial cells.

15. The method of any one of claims 1 to 14 wherein the one or more spheroids have a diameter of about 500 to about 600 microns.

16. The method of any one of claims 1 to 14 wherein the one or more spheroids have a diameter of about 450 to about 500 microns.

17. The method of any one of claims 1 to 16 wherein the one or more spheroids are cultured for at least 4 to 6 weeks before contacting with the one or more test compounds.

18. The method of any one of claims 3 to 17 wherein the fluorescent molecule comprises Calcium 3, Calcium 4, Calcium 5, Calcium 6, Fluo 3, or Fluo4.

19. The method of any one of claims 3 to 18 which further comprises contacting the wells with a cell membrane impermeant quencher.

20. The method of any one of claims 3 to 19 wherein the amount of change in fluorescence is compared to the fluorescence in a well with spheroids and the fluorescent molecule but no test compound.

21. A multi-well plate comprising one or more mixed human cell spheroids per well.

22. The plate of claim 21 wherein the one or more spheroids comprise neurons and astrocytes.

23. The plate of claim 21 or 22 wherein the one or more spheroids comprise heart, liver, kidney, pancreas, lung, endothelial or epithelial cells.

24. The plate of any one of claims 21 to 23 wherein the one or more spheroids comprise microglial cells or oligodendrocytes.

25. The plate of any one of claims 21 to 24 wherein the one or more spheroids comprise pericytes and endothelial cells.

26. The plate of any one of claims 21 to 25 wherein the one or more spheroids comprise endothelial cells, microglial cells, neurons, oligodendrocytic cells, or any combination thereof.

27. The plate of any one of claims 21 to 26 wherein the one or more spheroids comprise progenitors of neurons, astrocytes, heart cells, liver cells, kidney cells, pancreas cells, lung cells, endothelial cells, or epithelial cells.

28. The plate of any one of claims 21 to 27 wherein the wells comprise spheroids formed from different cells.

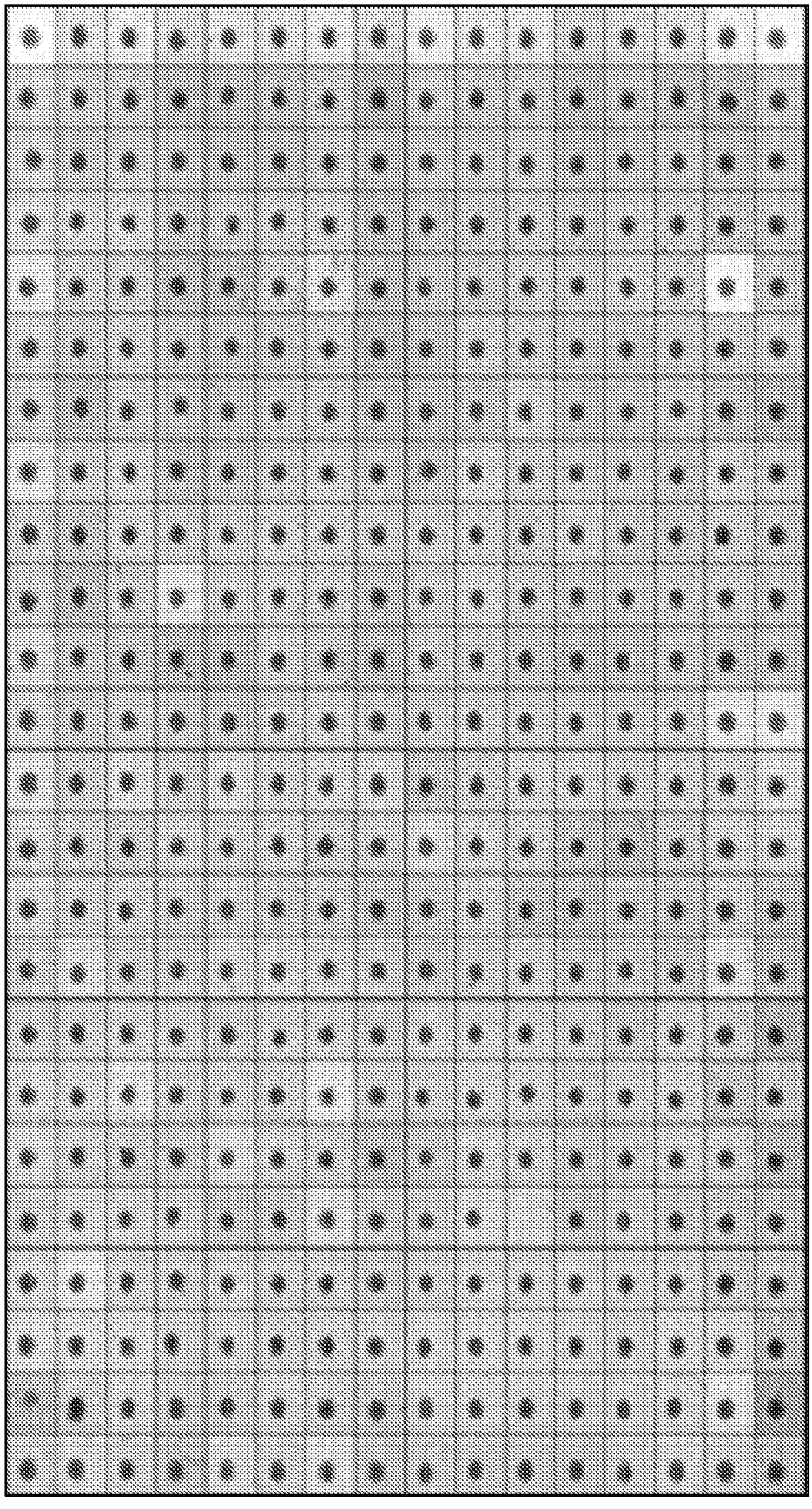

PLATE 10 WITH 384 WELLS, EACH HAVING A SPHEROID 20
384w MICROBRAIN 3D PLATE

Fig. 1

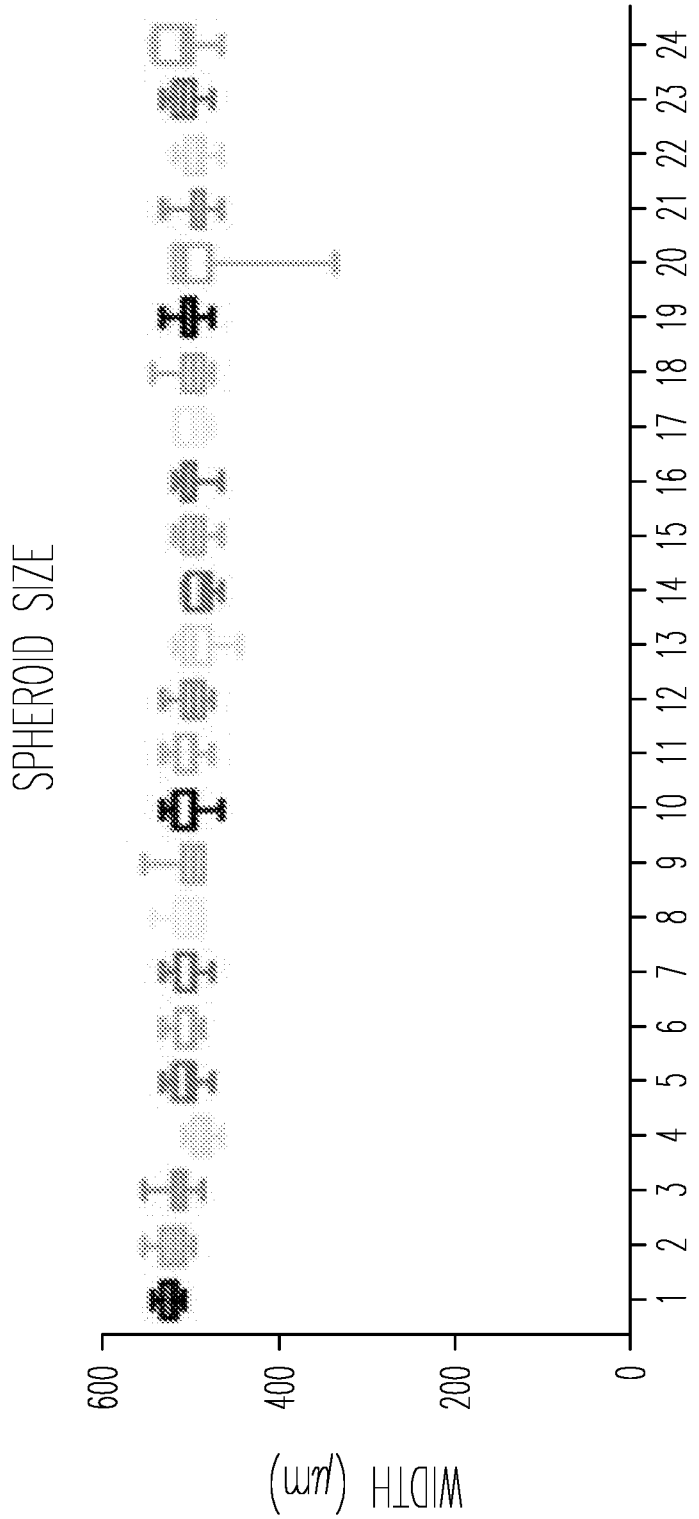

SPHEROID SIZE
WIDTH (μm)
600
400
200
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
COLUMN

Fig.2

SPHEROID 20

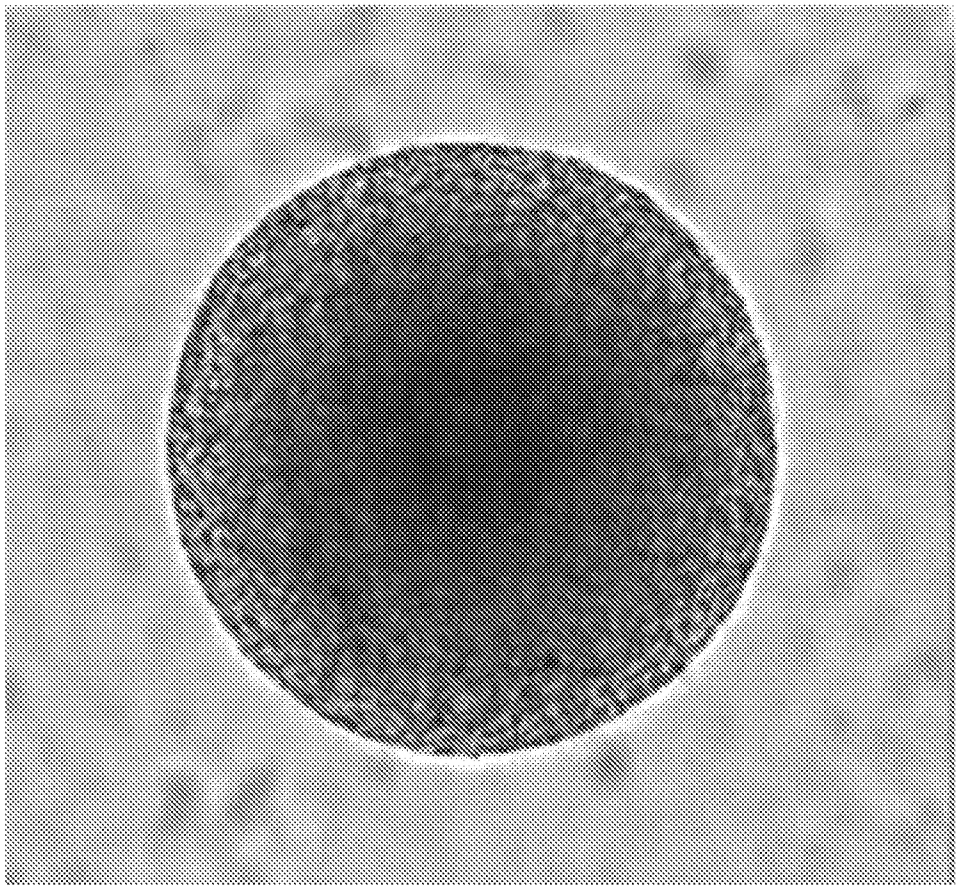
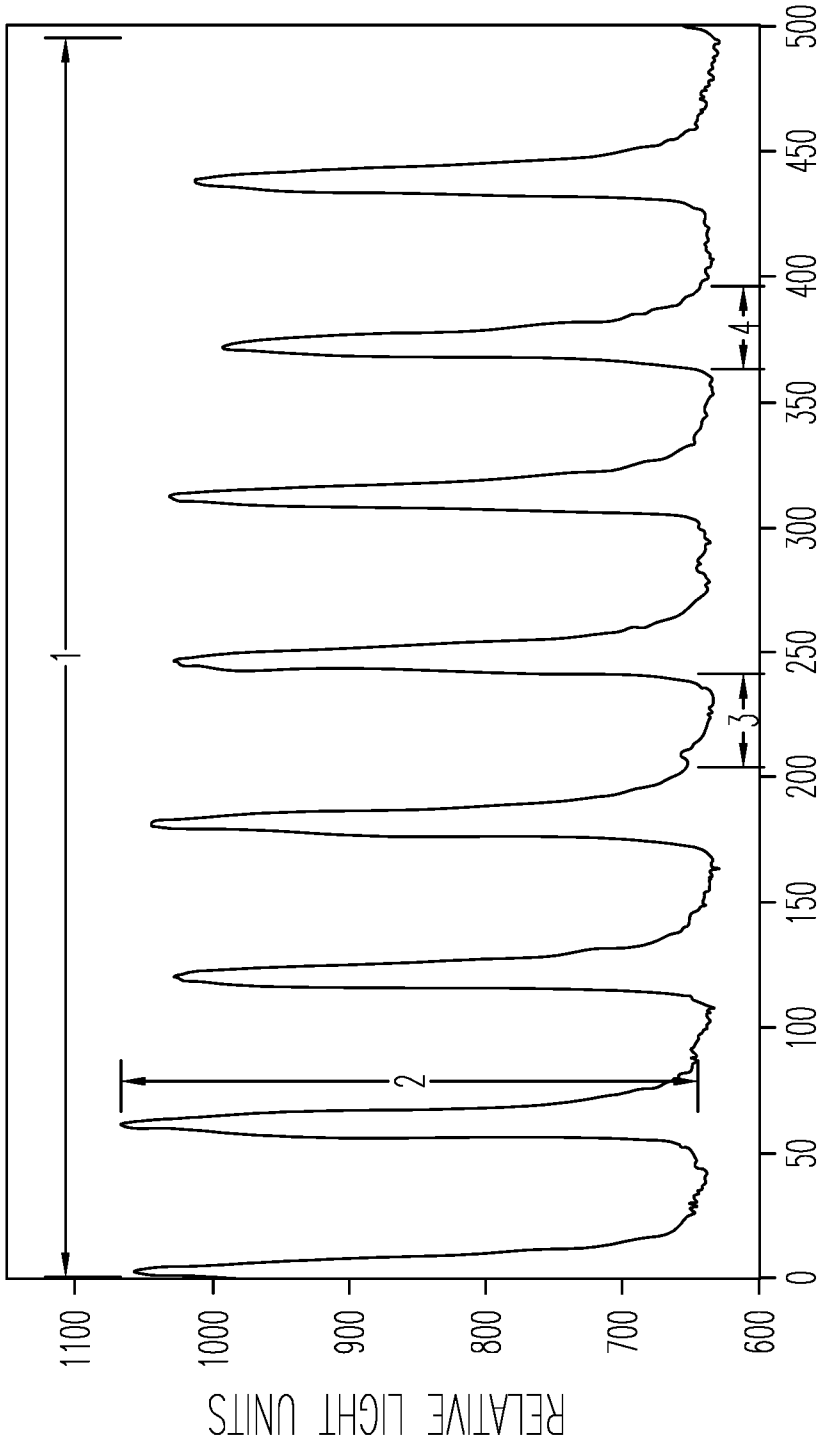


Fig. 3



GRAPH SHOWING 8 PEAKS 30

Fig. 4

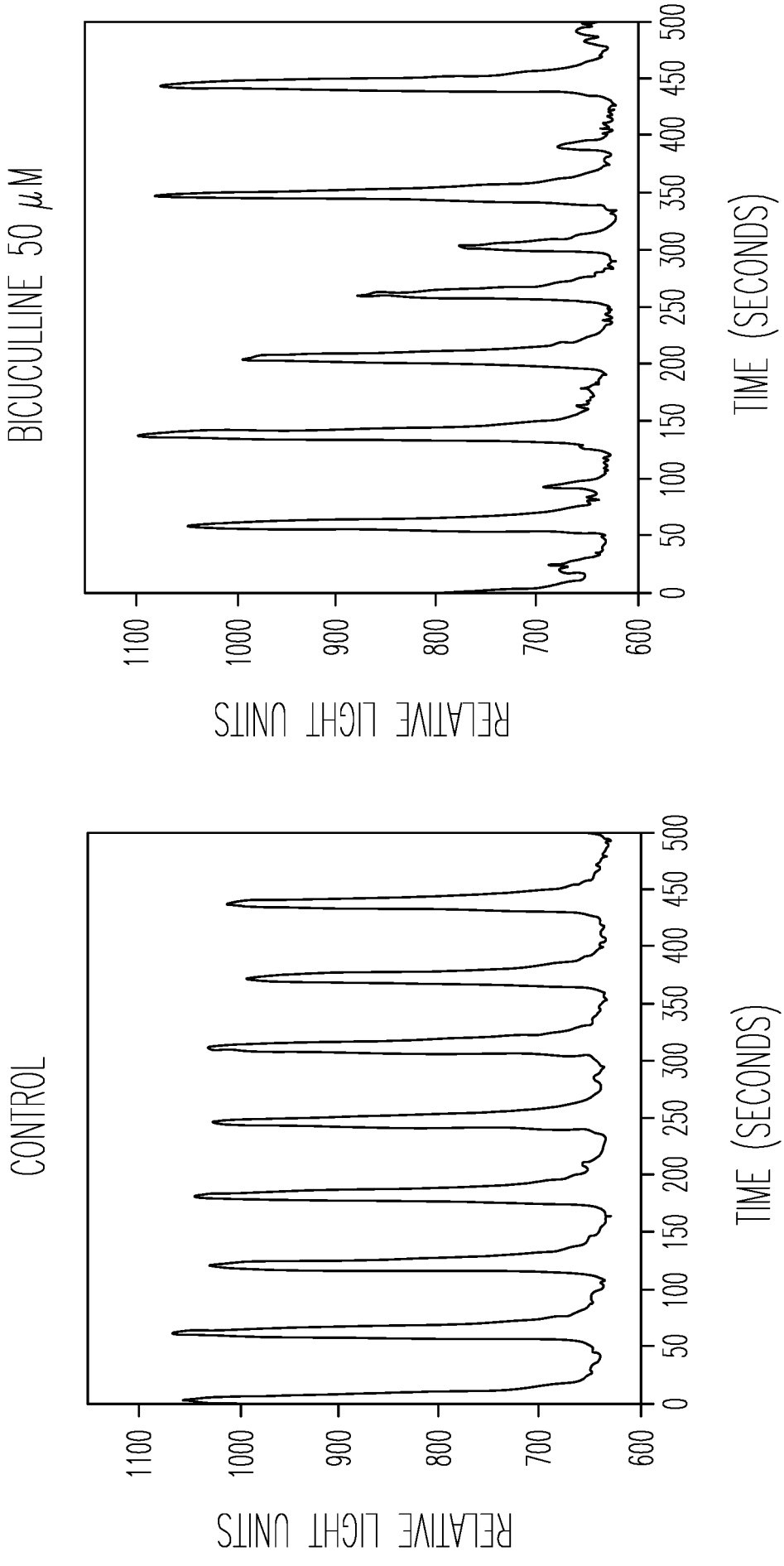
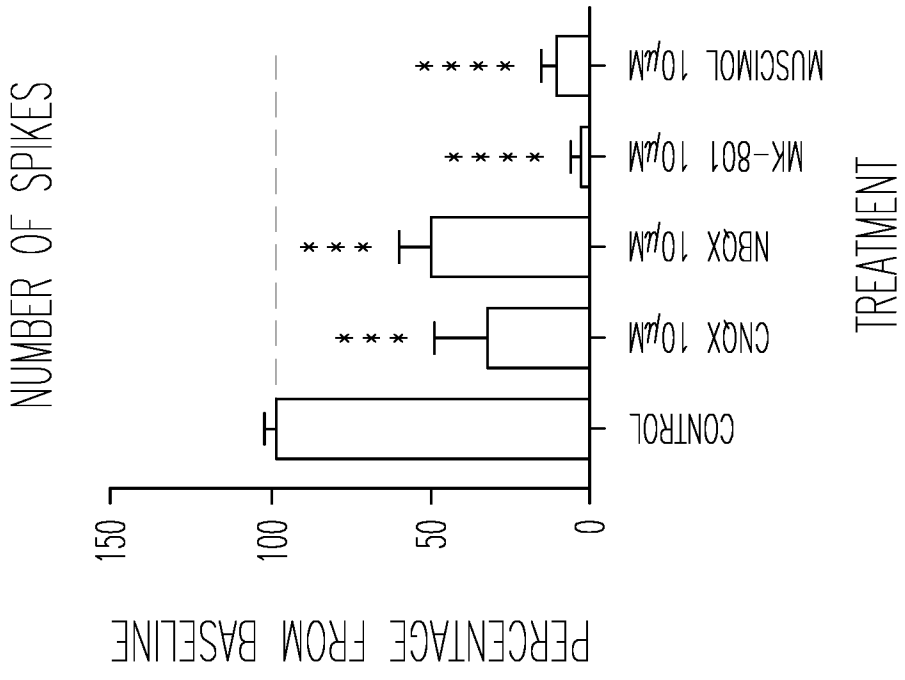

BICUCULLINE 50 μM
CONTROL
RELATIVE LIGHT UNITS
TIME (SECONDS)
600
700
800
900
1000
1100
0
50
100
150
200
250
300
350
400
450
500

Fig. 5

COMPOUND	MECHANISM OF ACTION
CNQX	POTENT AMPA/KAINATE RECEPTOR ANTAGONIST. ALSO ANTAGONIST AT GLYCINE MODULATORY SITE ON NMDA RECEPTOR COMPLEX
NBQX	POTENT, SELECTIVE AND COMPETITIVE AMPA RECEPTOR ANTAGONIST
MK-801	A POTENT, SELECTIVE AND NON- COMPETITIVE NMDA RECEPTOR ANTAGONIST
MUSCIMOL	POTENT $GABA_A$ RECEPTOR ANTAGONIST

Fig. 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/042105

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 C12N5/071 G01N33/68
ADD.

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)
G01N C12N

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	EP 2 974 751 A1 (UNIV SAGA [JP]; CYFUSE BIOMEDICAL K K [JP]) 20 January 2016 (2016-01-20) example 1; figs. 1-2; figs. 2-7	1-3,8, 15,16, 21-28 4-7, 9-14, 17-20

X A	WO 2016/115489 A1 (UNIV CLEMSON RES FOUNDATION [US]; MUSC FOUND FOR RES DEV [US]; UNIV CH) 21 July 2016 (2016-07-21) abstract; example 3; p. 28, par. 3; fig. 14	1-5,8, 18,21-28 6,7, 9-17,19, 20

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[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
22 October 2018	30/10/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hohwy, Morten

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/042105

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/115275 A1 (REGE KAUSHAL [US] ET AL) 27 April 2017 (2017-04-27)	1-5,8,9, 15,16, 18,21-28
A	example 7;par. 71	6,7, 10-14, 17,19,20

X	STEPHANIE M. RAVENSCROFT ET AL: "Cardiac Non-myocyte Cells Show Enhanced Pharmacological Function Suggestive of Contractile Maturity in Stem Cell Derived Cardiomyocyte Microtissues", TOXICOLOGICAL SCIENCES, vol. 152, no. 1, 28 April 2016 (2016-04-28), pages 99-112, XP055504282, ISSN: 1096-6080, DOI: 10.1093/toxsci/kfw069	1-5,8, 13-16, 21-28
A	abstract; p. 102, col. 1; figs. 3-4	6,7, 9-12, 17-20

X,P	OKSANA SIRENKO ET AL: "Phenotypic Assays for Characterizing Compound Effects on Induced Pluripotent Stem Cell-Derived Cardiac Spheroids", ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES, vol. 15, no. 6, 1 August 2017 (2017-08-01) , pages 280-296, XP055504291, US ISSN: 1540-658X, DOI: 10.1089/adt.2017.792 the whole document	1-28

X	TERRASSO ANA PAULA ET AL: "Novel scalable 3D cell based model forin vitroneurotoxicity testing: Combining human differentiated neurospheres with gene expression and functional endpoints", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 205, 5 January 2015 (2015-01-05), pages 82-92, XP029240251, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2014.12.011	21-28
A	abstract	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/042105

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:

see additional 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 an 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.:

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-20

A method of detecting the effect of a compound on cell spheroids.

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2. claims: 21-28

A multi-well plate comprising one or more mixed human cell spheroids per well.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/042105

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2974751 A1	20-01-2016	CN 105025939 A	04-11-2015
		EP 2974751 A1	20-01-2016
		JP WO2014141528 A1	16-02-2017
		US 2016022870 A1	28-01-2016
		WO 2014141528 A1	18-09-2014
WO 2016115489 A1	21-07-2016	US 2017369847 A1	28-12-2017
		WO 2016115489 A1	21-07-2016
US 2017115275 A1	27-04-2017	NONE	