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(54) Title:

**APPARATUS AND METHODS FOR CONTROLLING
CELLULAR DEVELOPMENT**

(57) Abstract:

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**APPARATUS AND METHODS FOR
CONTROLLING CELLULAR DEVELOPMENT**

According to one aspect and example, a method for facilitating cellular interactions in biological tissue provides controllable activation of a selected type of stem cell among a plurality of cell types present in the tissue. The method includes various steps including the introduction of a microbial opsin into a region of the tissue that includes a selected type of stem cell, by expressing the microbial opsin in the stem cell. A light source is then introduced near the stem cell, and the light source is used to controllably activate the light source to direct pulses of illumination from the light source to the selected type of stem cell, for selectively controlling the growth and development of the stem cell in a manner that is independent of the growth and development of the other types of cells.

Figure 1

APPARATUS AND METHODS FOR CONTROLLING CELLULAR DEVELOPMENT

Related Patent Documents

5 This patent document claims the benefit, under 35 U.S.C. § 119(e), of
U.S. Provisional Patent Application Serial No. 61/132,163 filed on June 17, 2008 and
entitled "Control of Cellular Interactions In Engineered Tissue," and of U.S. Provisional
Patent Application Serial No. 61/093,086 filed on August 29, 2008, and entitled
10 "Arrangements, Methods and Compositions Involving Modulation of Embryonic Stem
Cell Differentiation with Automated Temporally Precise Optogenetic Stimulation;"
the underlying provisional applications and respective Appendic(es) are fully
incorporated herein by reference. This patent document also relates to, and fully
incorporates by reference, the following underlying patent documents: U.S. Patent
Application Serial No. 11/459,636 filed on July 24, 2006 (STFD.169PA), PCT Patent
15 Application Serial No. PCT/US2008/050628 filed on January 9, 2008 (STFD.150PCT),
and U.S. Patent Application Serial No. 12/187,927 filed on August 7, 2008
(STFD.167PA) (*e.g.*, discussion in connection with FIGs. 1-5).

Field of the Invention

20 The present invention relates generally to methods, devices and systems for the
growth and development of cells and/or tissue.

Background

25 Naturally developing tissue is intrinsically of a multi-cell-type nature. A
substantial portion of cultured stem cells that are implanted, die without reaching maturity
or integrating themselves into a functional tissue system. The odds of survival and
functional integration increase when cultured cells are allowed to develop along side of
their natural companion cells. In many cases, the number of surviving cells may be
improved by growing glial cells and endothelial cells or fibroblasts along with neurons.
This generally holds true both in culture, and after implantation.

30 Tissue culture, involving the growth of tissues and/or cells separate from the
organism, is typically facilitated by use of a liquid, semi-solid, or solid growth media,
such as broth or agar. When intended for implantation as a solid organ, *e.g.*, in the
context of regenerative medicine, a suitable matrix is usually required. Even with the
appropriate immature cells (*e.g.*, stem cells) in place, development into function, and/or

implantable tissue does not occur spontaneously. In the specific case of neural tissue, for example, brain, axonal and dendritic sprouting is shaped by activity of the various cells in the milieu. In this way, local cellular environments are crucial in the regulation of neurogenesis. Empirically, scientists have evidenced that hippocampal cell co-culture promotes hippocampal neurogenesis, and that adult NPCs grown in an environment non-permissive for neurogenesis are unable to respond to excitation. These cells communicate with one another, *e.g.*, via chemical, molecular and electrical signals. Frequently, chemical or molecular signaling is triggered by electrical signaling; for example an endocrine cell releasing a growth factor when electrically stimulated.

Activity-dependent competition frequently occurs in this context. For example, more active neurons from one brain region may overgrow regions occupied by less active neurons. Conversely, limiting activity in a brain region during development results in functional deficits. Electrical signaling and molecular signaling are the most common approaches by which cells in culture control mutual behavior within the milieu.

Electrical signaling is an important part of nerve cell development and for many other types of cells including endocrine cells and muscle cells. The application of electrical pulses to neuronal progenitor cells (NPCs) causes them to evolve from generic sphere-like structures into mature neurons, sprouting axons and dendrites along the way, and establishing electrical connections with other neurons.

Chemical/molecular signaling is frequently triggered by electrical signaling. For example, adult neurogenesis and maturation of NPCs is greatly enhanced by excitatory stimuli and involves Cav1.2/1.3 channels and NMDA receptors. These Ca^{2+} influx pathways are located on the proliferating NPCs, allowing them to directly sense and process excitatory stimuli. The Ca^{2+} signal in NPCs leads to rapid induction of a gene expression pattern that facilitated neural development. This leads to synaptic incorporation of new neurons into active neural circuits. Another example is endocrine cell releasing a growth factor when electrically stimulated, but may also be triggered by other molecular or chemical signals. Nerve growth factor (NGF) is secreted by cells surrounding a developing neuron, such as glial cells, and is critical to the development and long-term survival of neurons. Nerve growth factor (NGF), is a small protein secreted by glial cells as well as by some neurons, and induces the differentiation and survival of target neurons. NGF binds to and activates its high affinity receptor (TrkA), and a low-affinity receptor (LNGFR), and promotes neuron survival and differentiation.

Conversely, molecular modifications of NGF such as proNGF can elicit apoptosis. Brain-derived neurotrophic factor (BDNF) is released from cells including fibroblasts and endothelial cells (such as those within capillaries), and serves to promote growth and development of neurons, including axonal and dendritic sprouting. Deficient expression
5 of BDNF not only impairs the development of neurons, but also impairs the development of capillaries and the survival of endothelial cells themselves. NGF, BDNF and neurotrophin-3 bind to the neurons bearing tyrosine kinase (trk) receptors trk A, trk B and trk C. Vascular endothelial growth factor (VEGF)-D is a member of the VEGF family of angiogenic growth factors that recognizes and activates the vascular endothelial growth
10 factor receptor (VEGFR)-2 and VEGFR-3 on blood and/or lymphatic vessels. Neuropilin-1 (NRP-1), for example, is one of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptors that is involved in normal vascular development.

Electrical and chemical/molecular signaling has limitations, however. For
15 example, electrical stimulation is rather agnostic to the types of cells that it activates. In brief, an electric field of a given distribution displays relatively low preference with respect to the type of cells which they affect. Electrodes indiscriminately influence the behavior of activate neurons, glia, endocrine cells, muscle cells, and even the growth of bone within the stimulated area. As a result, physical proximity of an electrode pole to a
20 given cell may be the single largest determining factor as to whether or not it is affected. Because of these limitations, it is generally not possible to exclusively affect a specific class of cell in heterogeneously populated tissue.

Intercellular molecular signaling, although frequently cell-type specific, is often not readily modified artificially in a physically tightly knit cell culture environment,
25 which frequently resists permeation of required growth factors, particularly in the absence of efficient capillary development. Proper and/or ideal distribution of chemical and molecular signaling agents including K⁺, BDNF, NGF, and VEGF may be best achieved using the cells that natively produce these agents, in their natural spatial configurations with respect to the target cells. Because molecular signaling is frequently triggered by
30 electric signals to the source cell, such signaling is subject to the non-specify of electrical activity within the milieu.

There are a number of challenges to successful production of a cultured neuronal tract using stem cells (either adult stem cells or embryonic stem cells). These challenges

have included issues emanating from maturing stem cell arrays remaining in evolution continuously, and connections being made between them early in their life where the connections may or may not be maintained as they develop further. Some method of ongoing functional reinforcement, either natural or artificial, is likely necessary for the long term viability of a cultured tract.

Efforts continue toward the goal of facilitating the consistent sprouting and growth of dendrites and axons in a predictable direction, as present studies show their natural development tendency to be lateral and/or randomly-directed growth.

10

Summary

The present invention is exemplified in a number of implementations and applications, some of which are summarized below.

In certain regards, the present invention is directed to providing mechanisms and methodology for individually and separately controlling the activity of specific cell types within a mixed tissue culture milieu, in order to direct optimal development of that tissue.

Certain aspects of the present invention are directed to using the intrinsic properties of axons and dendrites to facilitate the controlled development of young neurons. As specific examples, dendrites and axons have different associated chemo-attractants, temporal properties (axons grow faster than dendrites), and physical dimensions (axons are longer and thinner than dendrites). These properties may provide means by which one shape the development of young neurons.

According to one example embodiment, a method for facilitating cellular interactions in biological tissue or cell culture provides controllable activation of a selected type of stem cell among a plurality of cell types. The method includes introducing a microbial opsin into a region of the tissue or cell culture that includes a selected type of stem cell, by expressing the microbial opsin in the stem cell. A light source is then introduced near the stem cell, and the light source is used to controllably activate the light to direct pulses of illumination from the light source to the selected type of stem cell, for selectively controlling the growth and development of the stem cell in a manner that is independent of the growth and development of the other types of cells.

Also consistent with the present invention, one specific embodiment is directed to providing for discrete communication with specific cell types within a mixed-cell culture milieu, whereby cells of individual types and individual roles in tissue development can

be governed. Each of these selected cell types can thereby be induced to release their specific products on demand, as determined manually, or by a computer system. This approach is intended to enable maximal control of virtually all aspects of a tissue being cultured or engineered.

5 Another specific embodiment provides for artificially growth of a tissue within a predetermined spatial and geometric configuration. For example, a longitudinally-extending system of electrically interconnected neurons which propagates signals detected at one end of the system, and outputs a corresponding signal at the other end. An artificially-produced neuronal tract could serve as a replacement for a damaged
10 neuronal tract, for example in an injured human brain or spinal cord.

 Another specific embodiment is directed to a method for internal pacing of portion of a brain, *e.g.*, hypoactive or hyperactive portion of a brain being internally paced, while using another portion of the brain as the controller (*e.g.*, as opposed to an external source like a DBS pulse generator).

15 Yet another specific embodiment is directed to retaining stem cell somas enclosed within a predetermined range of migration. This aspect of the present invention recognizes that stem cells can escape from their implanted location, particularly embryonic stem cells, and therefore may seed themselves as cancerous tumors within the body.

20 Applications include the culturing of tissue, and the continued nurturing stimulation applied to an area of cells implanted *in vivo*. The specification details the application of an optogenetic approach which endows specific targeted cell types with a privileged channel of communication. Non-targeted cell types remain unaffected by that particular wavelength of light, but may be sensitized to a different wavelength or signal.

25 Embodiments consistent therewith specifically regard the regulation of neural tissue development suited for spinal cord or brain injury repair. However the same general principles of independent control of different cell types within the developing tissue apply to heart, liver, pancreas, kidney, bone and other tissues of the body, in culture or implanted *in vivo*.

30 Another aspect of the patent invention is directed to use and introduction of a microbial opsin into embryonic stem cells and the development of optogenetic technology for stem cell engineering applications, with a novel automated system for noninvasive

modulation of embryonic stem cell differentiation employing fast optics and optogenetic control of ion flux.

According to yet another embodiment, the present invention is directed to CNS (central nervous system) disease/behavior treatment (applicable, e.g., to Parkinson's Disease, stroke, and spinal cord injury) by functionalizing neurons to integrate into the host after intracerebral transplantation. To this end, the present invention is directed to stem cell therapy for CNS disease/behavior treatment wherein differentiated cells are generated, integrated into native neural circuitry and then controlled selectively by light.

The above summary of the present invention is not intended to describe each illustrated embodiment or every implementation of the present invention. The figures and detailed description that follow more particularly exemplify these embodiments.

Brief Description of the Drawings

The invention may be more completely understood in consideration of the detailed description of various embodiments of the invention that follows in connection with the accompanying drawings, in which:

FIG. 1 illustrates a system, according to an embodiment of the present invention, involving classes of cell types that function in a coordinated fashion during tissue growth, development, activity and maintenance, for selective activation (*e.g.*, stimulation or suppression), and their detection of their activity.

FIGs. 2a and 2b illustrate an assembly of biological and synthetic components, and stimulation means for multichannel stimulation tissue culture, according to an embodiment of the present invention;

FIGs. 3a and 3b illustrate a system for culturing tissue samples in accordance with the present invention;

FIG. 4 illustrates a system, also in accordance with the present invention, that uses multiple transductions used upon a cell, and shows activity feedback mediated by the activity of a secondarily impacted second cell type;

FIG. 5 illustrates a multichannel stimulation and monitoring system, also in accordance with the present invention, suitable for governing tissue development either in culture or post-implantation;

FIG. 6 is a schematic illustration of *in vivo* implantation and integration of cultured tissue into a living organism whereby development may be facilitated in accordance with the present invention; and

FIGs. 7-11 depict images and charts showing results of experimental implementations in accordance with the present invention.

While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described. On the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

Detailed Description

The present invention is directed to methods and apparatus for culturing and promoting the growth of stem cells, such as embryonic stem cells, in biological tissue. The present invention has been found to be particularly suited for use in arrangements and methods dealing with growth of stem cells in neural networks. While the invention is not necessarily limited to such biological environments, various aspects of the invention may be appreciated through a discussion of various examples using this context.

Consistent with one example embodiment of the present invention, a method for facilitating cellular interactions in biological tissue or cell culture provides controllable activation of a selected type of stem cell among a plurality of cell types whether or not present in the tissue or cell culture. The method includes introducing a microbial opsin into a region of the tissue or cell culture that includes a selected type of stem cell, by expressing the microbial opsin in the stem cell. A light source is then introduced near the stem cell, and the light source is used to controllably activate the light to direct pulses of illumination from the light source to the selected type of stem cell, for selectively controlling the growth and development of the stem cell in a manner that is independent of the growth and development of the other types of cells.

Figure 1 illustrates several classes of cell types which function in a coordinated fashion during tissue growth, development, activity and maintenance. These cell types may be selectively stimulated or suppressed, and their activity may be detected, for example by the array of colored LEDs and selective-color-filtered photodiodes. Each LED and photodiode are controlled by separate channels coupled to a computer. Computer controller 100 sends and receives inputs and outputs via multichannel driver 101, which in turn, communicates with each cell via transducers (LEDs 187, 177 and 156, and photodiodes 167 and 147), connected via multichannel cable 150. LED 187 emits

light 186 which produces ion channel modulation in glial cell 185 via ChR2. This produces a release of neurotrophic chemicals 184 (for example BDNF), which are received by neuron 120, thereby inducing growth and development in neuron 120. Neuron 120, as a product of its growth, releases tropic chemicals such as vascular-
5 endothelial growth factor (VEGF), which is received by capillary 145, and promotes growth of the network of which capillary 145 is a part. LED 177 emits light 176 which produces ion channel modulation in neuron 175. Band-filtered photodiode 167 receives light 166 of the wavelength emitted by an indicator (such as voltage dye) released from neuron 165 in response to action potential 164. LED 157 emits light 156 of a wavelength
10 which produces ion channel modulation in fibroblast 155. Band-filtered photodiode 147 receives light 146 of the wavelength emitted by an indicator for example those characteristic wavelengths emitted, for example, by Fura-2 or RH1691. Neuron 120 has axon 122, which communicates via synapse 129 with second neuron 165 with axon 175. Neurons 120 receive metabolic support from glial cell 185. Glia cell 185 draws nutrition
15 from end-feet 186 on capillary 145, and delivers nutrition to neuron cell 120 via end-feet 187. Microglia 140 (representative sample shown) are dispersed throughout. Ca^{2+} influx pathways are located on the proliferating NPCs, allowing them to directly sense and process excitatory stimuli. The Ca^{2+} signal in NPCs leads to rapid induction of a gene expression pattern that facilitated neural development. This leads to synaptic
20 incorporation of new neurons into active neural circuits. Another example is endocrine cell releasing a growth factor when electrically stimulated, but may also be triggered by other molecular or chemical signals. Nerve growth factor (NGF) is secreted by cells surrounding a developing neuron, such as glial cells, and is critical to the development and long-term survival of neurons. Nerve growth factor (NGF) is a small protein secreted
25 by glial cells as well as by some neurons, and induces the differentiation and survival of target neurons. NGF binds to and activates its high affinity receptor (TrkA), and a low-affinity receptor (LNGFR), and promotes neuron survival and differentiation. Conversely, molecular modifications of NGF such as proNGF can elicit apoptosis. Brain-derived neurotrophic factor (BDNF) is released from cells including fibroblasts and
30 endothelial cells (such as those within capillaries), and serves to promote growth and development of neurons, including axonal and dendritic sprouting. Deficient expression of BDNF not only impairs the development of neurons, but also impairs the development of capillaries and the survival of endothelial cells themselves. NGF, BDNF and

neurotrophin-3 bind to the neurons bearing tyrosine kinase (trk) receptors trk A, trk B and trk C. Vascular endothelial growth factor (VEGF)-D is a member of the VEGF family of angiogenic growth factors that recognizes and activates the vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 on blood and/or lymphatic vessels.

- 5 Neuropilin-1 (NRP-1, for example, is one of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptors that is involved in normal vascular development. Optogenetic methods may be used to trigger the release of compounds such as BDNF, NGF, GDNF and VEGF.

One function of G-Proteins is to mediate the process by which a stimulus upon a
10 cell impacts the response of that cell; for example, the timing of electrical spikes delivered upon a neuron may or may not translate into the emergence of excitatory post-synaptic potentials, depending upon G-protein activities. G-proteins may carry out their roles by using various subordinate mediators. G-proteins such as Gx and Gq may be induced (by optical or pharmacological stimulation) so as to release factors such as
15 BDNF, NGF, GDNF and VEGF. Stimulation of the G-protein may be accomplished in a cell-type-specific manner (for example using cell-type-specific genetic targeting and optogenetic stimulation methods as described in one or more of the underlying provisional patent documents and as described in Airan R.D., Thompson K.R., Fenno L.E., Bernstein H., Deisseroth K., *Temporally Precise in vivo Control of Intracellular
20 Signaling*, Nature, 2009 Apr 23, 458(7241):1025-9, Epub 2009 Mar 18. When this is done, the regulation and control of a cell's response level to such factors applies only to the selected type of cell, and not to other adjacent populations within a tissue culture, neural circuit, animal, or patient. G-proteins may also be used to control the release of dopamine, norepinephrine, serotonin, vasopressin, oxytocin, and other neurotransmitters
25 and hormones. Control of G-protein activity, thereby permit control of cellular differentiation, and which neural circuits are turned on or off at a given time.

Methods for external readout of levels of cellular activity within a network are known in the art. As described in Knopfel *et al.*, *Optical probing of neuronal circuit dynamics: genetically encoded versus classical fluorescent sensors*, Trends Neurosci.
30 2006 Mar 29, 3:160-6, such methods include use of non-protein calcium sensors such as Fura-2, Oregon green 488 BAPTA-1, and X-Rhod-5F; genetically-encoded calcium sensors, such as yellow cameleon 3.6, G-CaMP2, Camgaroo-2 and TN-L15; non protein voltage sensors such as di-4-ANEPPS and JPW3028; and hybrid voltage sensors such as

hVOS, genetically-encoded sensors such as FlaSh, SPARC and VSFP1. Additionally, absorbance-based measures of calcium flux such as RH-155 may be used by means known in the art.

5 Methods of providing readout regarding expression of cell products and the subpopulations of cells that produce them with an antibody linked to a fluorescent dye. For example, for gauging developmental stage of cellular development, one may use nestin staining (*see, e.g.*, underlying U.S. provisional application No. 61/093,086).

10 Additionally, both size and morphology degree of differentiation in developing cells may be assessed and read out using automated image analysis software and systems. One example is a microscopy system built upon the PERL-based OME server project at Open Microscopy Environment (www.openmicroscopy.org), which implements image-based analysis of cellular dynamics and image-based screening of cellular localization or phenotypes. Another example of software readout may be based upon BD IPLab Advanced Image Analysis Software (BD Biosciences, Rockville, MD). Other methods of
15 providing readout regarding cellular activity are known in the art, and include spectroscopy (absorbance and transmittance), functional magnetic resonance imaging (such as use of the BOLD effect), and positron emission tomography. Readout on cellular metabolic activity may also be obtained via electronic chemical “sniffers” which react to the presence of gasses such as carbon dioxide.

20 FIG. 2a illustrates an assembly of biological and synthetic components, and stimulation means for multichannel stimulation tissue culture within an engineered tissue culture matrix. Pulse generator 201 provides power to LED 225 and LED 226, each of which emit a different spectrum and parameters, while power 224 and ground 223 provide the current flow required. LED 225 may, for example, emit blue light at 50 Hz,
25 while LED 226 emits yellow light at 100 Hz. The electronics described in FIG. 1 are simplified for illustrative purposes. In practice, multiple pulse generators operating separately are used for the implementation of separate channels, and these channels may be activated independently depending upon readout data entering the system, as will be described in subsequent figures. Neuronal progenitor cells (NPCs, neural stem cells) 205,
30 glial progenitor cells (GPCs, glial stem cells) 206 and vascular progenitor cells (VPCs, vascular stem cells) 207 are added to cellular growth media 208. All are held within encapsulating porous membrane 217, and against porous membrane 210, enclosed by the addition of porous membrane 204. Porous barrier membrane 209 containing membrane

pores 210 serves to prevent cell migration out of the engineered matrix, and prevents clumping in other portions of the engineered matrix. Porous membrane 209 may be composed of materials such as polyethylene terephthalate porous membrane. Generally, pores 210 are of a diameter between approximately 3 and 7 μ . At these dimensions, pores 10 are generally too small for stem cell bodies (soma) to pass through, but large enough for dendrites and axons to pass through. Porous membrane 209 serves as an anchoring layer (either above, below, above and below or enclosing around) which restricts cell migration before and during the growth of axons and dendrites, and provides easy means for removal of the cells from the culturing apparatus prior to implantation. In an adjacent but separated compartment of the engineered matrix, NPCs 211 are added to cellular growth media 212, and more glial precursor cells and vascular precursor cells 207 may be added. Porous barrier membrane 213 serves to close off this compartment of the matrix to prevent migration or clumping, as was previously done with porous membrane 210. In a subsequent compartment, NPCs 214 are again added to cellular growth media 15 215, and the additional cell types previously specified. Encapsulating porous membrane 216 containing membrane pores 217 encloses the entire engineered matrix described. Encapsulating porous membrane 216 may be composed of materials such as polyethylene terephthalate porous membrane. Generally, pores 217 are of a diameter between approximately 3 and 7 μ . At these dimensions, pores 217 are generally too small for stem 20 cell bodies (soma) to pass through, but large enough for dendrites and axons to pass through. These pores 217 also permit physiological gas exchange, and the influx of nutrients from microvascular structures and glial cells located outside of luminous membrane 216. Cell and media compartment 218, 219, 220, 221 are analogous to the compartmentalized cell groups described above, and likewise serve to prevent migration 25 or clumping.

Under specific conditions known in the art, a variety of cells of various lineages may be induced to produce any of a variety of products or growth factors. For example, neurons themselves may secrete BDNF, as well as gastric hormones (such as vaso-intestinal peptide (VIP) or somatostatin), much like endodermally-derived cells normally 30 do. In an alternative embodiment, neural stem cells or pluripotent stem cells or induced pluripotent stem cells (iPS) (Takahahi *et al.*, Yu *et al.*) may be used in place of more differentiated counterparts, with some portions acquiring, for example, a neuronal path of development, and others assuming, for example, a vascular path of development.

FIG. 2B illustrates in a generic manner, how two portions of the brain or body, region 260 and region 270, respectively, may be functionally connected or re-connected using cells or tissues grown in accordance with the present invention. In the case of the brain, regions 260 and 270 may represent two brain nuclei, the natural connection
5 between which has been severed, for example by a cerebrovascular accident. In the case of a spinal cord injury, regions 260 and 270 may represent the brain and a formerly paralyzed muscle, respectively. In the case of peripheral nerves, 260 and 270 may represent a spinal cord ganglion and a deafferentated hand, respectively. In the case of a cardiomyopathy, regions 260 and 270 may represent the vagus nerve and newly
10 regenerated heart tissue, respectively. Cells 265 may be neural, glial, and vascular cells or precursor (stem) cells, and are held in place by artificial matrix material 266, such as a porous polyethylene terephthalate film. Pulse generator 280 provides power to LED 285 to produce light emissions which fall upon cells 265.

Also in accordance with the present invention, Figures 3a and 3b show a system
15 for efficiently culturing numerous tissue samples. In a more particular implementations thereof, the system of FIG. 3a is a high-throughput multiwell system for efficiently culturing numerous tissue samples in parallel. FIG. 3a shows a multichannel emitter-detector unit as suited to the present invention. Multichannel emitter-detector unit 300 includes LED 315, phototransistor 325, phototransistor 335, LED 346 and LED 356, and
20 is placed over tissue culture well 305, containing cells 310, 320, 330, 340 and 350. LED 315 emits a specific wavelength band of light 316 which is received by type-X cells 310. LED 345 emits a specific wavelength band of light 346 which is received by type-Y cells 350. LED 355 emits a specific wavelength band of light 356 which is received by type-Z cells 340. Phototransistor 325 receives a specific wavelength band of light 326 which is
25 emitted by type V cells 320. Phototransistor 335 receives a specific wavelength band of light 336 which is emitted by type-W cells 330. In one embodiment, cell type V may be a neuron, cell type W may be an astrocyte, cell type X may be an astrocyte, cell type Y may be a fibroblast, and cell type Z may be a pancreatic beta cell. Cell types are referenced with variables V, W, X, Y, Z, in order to emphasize the diversity of cell types that are
30 amenable to this method of control. Furthermore, any of these variable may be of the same type as that represented by another variable. For example, a type V cell could be identical to a type X cell.

FIG. 3b show a high-throughput multiwall incubation control system for efficiently culturing numerous tissue samples in parallel in accordance with the present invention. In FIG. 3A, environmental control chamber 390 contains culture plate actuators 380, and culture plates 365, each containing tissue culture wells 370 (representative example). Multichannel emitter/detector unit 360 (representative example) is analogous with multichannel emitter/detector unit 300 of FIG. 3a, and is arranged into arrays of emitter/detector units 360, each of which is controlled by computer 375. Each multichannel emitter/detector units 360 sends stimuli and receives readouts including feedback from the stimuli, from the developing tissue in wells 370. Stimulation instructions and readout values are sent and received, respectively, by computer 375. Appropriate environmental conditions are maintained by heater 391, humidifier/gas mixture control 392, and thermostat 393, as coordinated by computer 375.

FIG. 4 illustrates the use of multiple transductions used upon a cell, and shows activity feedback mediated by the activity of a secondarily impacted second cell type. Gene 402 imparts light-sensitivity upon a host cell, for example the manner in which ChR2 creates light sensitivity in neurons. Gene 412 causes cells to give off light when they undergo a given physiological process. For example, the florescent agents described in Knopfel *et al.* 2006, causes neurons to give off light when they depolarize. Other examples might include a substance that effervesces light when it receives a given hormone (*e.g.*, BDNF) or neurotransmitter, or alternatively, gives off light when it secretes a given substance, such as VEGF. Gene promoter 401 acts to promote gene 402, and gene promoter 411 acts to promote gene 412. As a result 405 of this promotion, cell 420 physiologically responds to light of wavelength band 421 which is emitted from LED 420 as determined by electronic control signals detailed in FIG. 3. The response to this light may include self-recognized responses 421 (for example enhanced axonal and dendritic development), and externally recognizable responses (425), for example the release of vascular-endothelial growth factor (VEGF), or the promotion of axonal and dendritic development in an adjacent developing nerve cell. Externally recognized response 425 is shown received by cell 430, which, in turn, produces self-recognizable responses 431 as well as light of wavelength band 441. This light emission, of course, is another form of externally recognizable response. Light of wavelength band 441 is received by photodiode 440, producing an electronic detection signal, as detailed in the description of FIG. 3.

FIG. 5 illustrates a multichannel stimulation and monitoring system, suitable for governing tissue development either in culture or in-vivo/post-implantation. The principal subunits are multichannel pulse output generator 504 and multichannel detection signal receiver 550 as controlled by computer 502. Multichannel pulse output generator 504 selectively sends signals to the output portion of the apparatus. When signals are pulsed from Channel 1 Output 505, through Channel 1 switching transistor 510, power 501 is conferred to channel 1 LED 512. Likewise, when signals are pulsed from Channel 2 Output 506 through channel 2 switching transistor 522, channel 2 LED 522 is illuminated. Likewise, when signals are pulsed from channel 3 Output 507 through channel 3 switching transistor 530, channel 3 LED 532 is illuminated. Multichannel detection signal input 550 receives signals from sensors which monitor area of tissue culture or implantation. Channel 4 Input 551 receives signals from channel 4 photodiode 561 when the latter is activated. Similarly, channel 5 Input 552 receives signals from channel 5 photodiode 562 when the latter is activated. Likewise, channel 6 Input 553 receives signals from Channel 6 photodiode 563 when the latter is activated. The above circuitry operates between power 501 and ground 508. Computer 502 contains a knowledge base, algorithms or protocols for sending stimulation signals through pulse output generator 504 and for modifying these signals in accordance with patterns of signals received by multichannel signal detection receiver 550.

FIG. 6 schematically represents the *in vivo* implantation and integration of cultured tissue into a living organism whereby development may be facilitated in accordance with the present invention. Two apparatuses are shown for this purpose within the figure; 2-dimensional grid array 660 (an array of emitters and detectors), and a 3-dimensional multi-surface depth emitter and detector probe 650. Grid array 660 has leads 665 while probe array 650 has leads 655. Intervention zones 630 are the designated sites requiring tissue repair or development. Intervention sites 630 sites may be damaged or otherwise insufficient areas of brain 600 at which immature cells are implanted. Alternatively cells native to or which have migrated to these areas by natural means may be responsive to stimuli from grid array 660 or probe array 650. Grid array 660 is best suited for governing the behavior of developing tissue on surfaces of brain 600, while probe array 650 is best for reaching sub-surface areas of tissue development. In an alternative embodiment, 600 may instead represent another organ of the body other than the brain. Shown in both intervention zones 630 are Type I cells 620 and 640

(implanted), and Type II cells 625 and 645 (implanted or native). In an alternative embodiment, the discrete channels of communication may be a non-native chemical or molecular substance. For example, neurons may be sensitized to an arbitrary molecule which does not naturally function to affect a neuron. This may be accomplished, for example, by gene insertion for a receptor for this arbitrarily selected molecule, with the receptor functionally tied to the desired output function of that cell type. In this new configuration, whenever that arbitrary molecule is introduced into the culture, that cell will react. In the example of a neuron, it would fire an action potential, or alternatively, become hyperpolarized. Because no other type of cell in the milieu is sensitive to the selected molecule, astrocytes and endothelial cells do not react.

Another aspect of the patent invention is directed to use and introduction of a microbial opsin into embryonic stem cells to develop optogenetic technology for stem cell engineering applications, with a novel automated system for noninvasive modulation of embryonic stem cell differentiation employing fast optics and optogenetic control of ion flux.

In one experimental embodiment, mouse embryonic stem cells (ESCs) were stably transduced with ChR2-YFP and purified by FACS. Illumination of resulting ChR2-ESCs with pulses of blue light triggered strong inward currents. These labeled ESCs retained the capability to differentiate into functional mature neurons, assessed by the presence of voltage-gated sodium currents, action potentials, fast excitatory synaptic transmission, and expression of mature neuronal proteins and morphology. Optically stimulating ChR2-ESCs during the first 5 days of neuronal differentiation, with high-speed optical switching on a custom robotic stage and environmental chamber for integrated optical stimulation and automated imaging, drove increased expression of neural markers. These data point to potential uses of ChR2 technology for chronic and temporally precise noninvasive optical control of embryonic stem cells both *in vitro* and *in vivo*, ranging from noninvasive control of stem cell differentiation to causal assessment of the specific contribution of transplanted cells to tissue and network function.

As another aspect of the present invention and useful alone or in combination with other aspects disclosed herein, optogenetic technology (*e.g.*, as described herein) may be used to selectively affect certain cell types, rendering target cell types sensitive to light while other cell types remain insensitive to light. In this manner, such a system effectively differentiates between various cell types. In this regard, development of one cell type can

be distinguished from other cell types by creating a viral vector in which a cell-type-specific promoter gene sequence sits immediately adjacent to the portion which codes for an opsin such as ChR2 or NpHR. As specific examples, glial cells may be targeted by use of a GFAP promoter; neurons in general by a Synapsin-I promoter; excitatory
5 neurons by a CaMK2-alpha promoter; inhibitory neurons by a VGAT promoter; endothelial cells by a TIE-1 promoter, and stem cells including progenitor cells by a nestin promoter.

EXPERIMENTAL RESULTS

10

Transduction of mouse ESCs with ChR2

To assess the potential of optogenetics in stem cells, mouse ESCs were transduced with a lentiviral ChR2-YFP-construct under the control of the EF1a promoter; after sorting for the top 5% based on YFP fluorescence intensity, we found that the population
15 doubling time and vitality of the resulting ChR2-YFP-ESCs did not differ significantly compared to non-transduced ESCs (not shown), and confocal microscopy demonstrated membrane localization of ChR2-YFP with high, uniform expression levels in the ESC population. ChR2-ESCs continued to express the embryonic stem cell marker SSEA1 and Oct4 (not shown), maintaining the undifferentiated state as did non-transduced control
20 cells. Electrophysiologically, the ChR2-ESCs displayed typical outwardly rectifying and passive currents, while illumination with blue light (470 nm, 500 ms pulse duration) evoked inward photocurrents (FIGs. 7a, 7b); steady-state photocurrents showed little inactivation while peak photocurrents showed inactivation and recovery with kinetics similar to that previously shown in neurons³⁰ (FIG. 7c).

25

The microbial opsins, including ChR2, require a chromophore (all-trans-retinal) to absorb incoming blue photons and gate the conformational change of the protein. A surprising finding in the development of microbial opsins for neurobiology was that mammalian neurons (but not invertebrate neurons) appear to have sufficient endogenous retinoids to allow ChR2 to function without addition of any chemical cofactors. If
30 optogenetics is to become a useful tool in stem cell engineering, it will be important to determine in stem cells the extent of dependence on exogenous chemicals like retinoids both in vitro and in vivo. No retinoids were added for the in vitro experiments described above; to further determine dependence or independence from exogenous retinoids in vivo, 5×10^5 ChR2-YFP expressing ESCs were stereotactically injected into the cortex of

healthy rats. One week after transplantation, animals were sacrificed and in acute slices, transplanted cells could be identified by YFP fluorescence. To test whether transplanted Chr2-ESCs could still respond to optical Stimulation, patch clamp recordings were conducted, revealing inward currents upon illumination with blue light (FIG. 7d) that displayed typical inactivation of the peak current and stability of the steady-state current. Together these data demonstrate that optogenetic interventions can be effective, well-tolerated, and independent of exogenous chemical cofactors in mammalian ES cells.

Differentiation of Chr2- ESCs

Intracellular Ca^{2+} is a major mediator of differentiation and survival in stem cells and their progeny, especially in neural lineages. Chr2 itself is a nonselective cation channel that directly allows Ca^{2+} entry into cells. Additional routes of photo-evoked Ca^{2+} entry could include activation of voltage-gated Ca^{2+} channels (VGCCs) by virtue of Chr2-induced membrane voltage changes. Notably, we find that mouse ES cells express four major VGCCs assessed by RTPCR and immunoreactivity (FIGs. 8a, 8b), and this supplementary mechanism for photoactivated Ca^{2+} entry could become increasingly potent as cells proceed down the neuronal lineage and develop hyperpolarized membrane potentials. Regardless, the known Ca^{2+} flux of Chr2 itself suggested the potential for optical control of stem cell processes.

We first verified that Chr2-ESCs were capable of neural lineage differentiation, using a retinoic acid-based neural differentiation protocol (FIG. 8c). At differentiation day 8, $40 \pm 10\%$ of the cells expressed the neural lineage marker nestin. By day 14, a dense network of β -3-tubulin-positive ESC-derived immature neurons could be detected, followed by expression of the mature neuronal cytoskeletal protein MAP2 and the vesicular glutamate transporter II (vGlutII). By day 28 the resulting Chr2-ESC-derived neurons displayed mature neuronal morphology, sodium currents, action potentials, and excitatory postsynaptic currents which could be blocked by excitatory synaptic transmission glutamate receptor antagonists CNQX and D-AP5 (FIG. 9a-d).

Optical modulation of neural differentiation

One challenge in deriving replacement tissues from ES cells is that the cell-type specification and phenotype consolidation processes, and therefore also the patterning and differentiation stimuli, take place over many days; to be applicable, optogenetic

stimulation must therefore be deliverable in chronic fashion. In designing the system to meet this challenge, it is also important to consider that since knowledge of the precise combinations and timing of signaling events required for stem cell differentiation is limited, a multiwell configuration would in principle be desirable, to allow for fast optical mapping of cell lines, conditions, and "differentiation space" in the laboratory. We therefore devised an automated multiwell optogenetic stimulation approach designed to precisely revisit and optically stimulate multiple regions of interest (ROIs) in defined patterns over extended periods of time (FIG. 10a).

ROIs in multiwell plates were user-defined in a custom GUI and their locations saved for rapid and reproducible access by a robotic stage (FIG. 10a). Stimulation parameters (excitation filter wavelength, optical switch pulse duration, and frequency/duty cycle of excitation) were set per configured parameters via the software-based equipment that controls the microscopic stage in the three spatial dimensions, and controls operation of the DG-4 optical switch which employs spinning galvanometers to deliver light with sub-millisecond precision (FIG. 10a). The microscope itself is surrounded by a climate controlled Plexiglass chamber wherein both temperature and CO₂-level are tightly regulated and temporally precise imaging can proceed in parallel with optical stimulation (FIG. 10a). Embryonic stem cells can be cultured and photostimulated in this environment rather than in a standard incubator for many weeks, allowing us to investigate the effect of optogenetic stimulation on the differentiation of embryonic stem cells in a controlled, reproducible manner.

In a typical experiment, ESCs were seeded in a 24 well plate, at a density of 100,000 cells/ml and 1ml/well. To directly capitalize on the advantages of the multiwell plate format, certain wells were seeded with native ESCs and others with Chr2-YFP ESCs; moreover specific wells were programmed to receive optical stimulation; finally, in combinatorial fashion, different wells within groups received different concentrations of differentiation factors (for example, the neural lineage factor retinoic acid at 0, 1, or 2.5 μ M). In this way differentiation space could be efficiently mapped while controlling for nonspecific effects related to the rig or to illumination. Cells were stimulated for 5 days with blue light (470 nm at 15 Hz for 10s) delivered every 60 min using a 10x objective. The survival and morphology of the cells was monitored using time-lapse imaging every 8 hours (FIG. 10b-g), also demonstrating the precision and accuracy of the automated setup in its ability to precisely revisit the same ROI.

To identify rapidly-acting effects of optical stimulation on ESC differentiation, cells were simultaneously assayed following the conclusion of stimulation (FIG. 8c). Immunostaining for the neural marker nestin followed by confocal analysis of fluorescence histograms was used to quantify neural lineage differentiation, along with
5 imaging of cellular nuclei using DAPI. FIGs. 11a and 11b show a 3D projection of two typical confocal z-stacks of single ROIs, displaying both DAPI (blue) and nestin (red) fluorescence. Optically stimulated cells consistently showed higher nestin immunoreactivity (FIG. 11b) compared to non-stimulated cells (FIG. 11a), while optical stimulation interestingly was ineffective in the absence of retinoic acid (RA) (FIG. 11c).
10 To quantify this effect, we generated fluorescence intensity histograms of all ROIs across all wells in each condition (resulting in more than 150 confocal images per condition). These intensity histograms revealed considerable differences between stimulated and nonstimulated ChR2-ESCs (FIG. 11d-h; $p < 0.01$, Kolmogorov-Smirnov test). We next conducted an experiment to test the possibility that the nestin distributions of unmodified
15 ("native") optically stimulated ESCs (FIG. 5g) and ChR2-YFP optically stimulated ESCs (FIG. 11e) could represent samples from the same distribution; after automated optical stimulation, repeated as in the above experiment and subsequent blinded analysis, we found that this hypothesis could be rejected ($p < 0.001$; two-tailed K-S $Z = 5.43$; FIG. 11e,g shows the observed increase in high levels of optically-induced nestin expression in
20 the ChR2-YFP cells). We calculated the mean nestin fluorescence intensity in each condition, and comparing optically stimulated with non-optically stimulated cells across all conditions revealed that only ChR2-YFP ESCs incubated with 2.5pM RA showed a significant optogenetically-induced increase in mean nestin expression ($p < 0.01$, two-tailed t-test; FIGs. 11f, 11h). In the presence of 1 μ M RA, a nonsignificant trend toward
25 higher nestin expression in the setting of optical stimulation was observed, while in 0 μ M RA no effect of optical stimulation was observed (e.g., FIG. 11c).

Accordingly, the present invention presents an application of optical control technology to stem cell engineering, and demonstrates the potential of the optogenetic approach by successfully expressing and driving the light-gated cation channel
30 channelrhodopsin-2 in mouse embryonic stem cells. We found that ChR2-YFP ESCs were viable and maintained the undifferentiated state, and also retained the capability to generate electrophysiologically mature neurons when differentiated. Moreover, pulsed illumination with blue light evoked precise and robust cation currents in ESCs, enabling

reproducible and predictable control of ion flux without requiring addition of chemical cofactors either in vitro or within intact brain tissue. By developing automated multiwell optogenetic stimulation tools, we were able to deliver optical stimulation in combinatorial experiments over extended periods of time with high spatiotemporal precision, and found
5 that optogenetic stimulation could modulate neural lineage progression in the presence of 2.5 μ M RA.

As specifically discussed in connection with the underlying provisional documents, depolarization has been reported in other studies to modulate neural differentiation processes in dividing cells, and indeed depolarization and calcium waves
10 have both been observed in proliferating GNS progenitors *in situ*; for example, in early CNS development, Momose-Sato *et al.* demonstrated spontaneous depolarization waves, and Kriegstein and colleagues observed calcium waves in cortical progenitors. Likewise in postmitotic neurons, depolarization plays additional important roles in CNS development, affecting spine development and synaptic plasticity. In connection with the
15 present invention, it is now believed that while the specific signal transduction cascades mediating the influence of membrane depolarization events in early development remains unclear, the Ca²⁺ and Ca²⁺ channels may play a key role and ChR2 is well suited to recruit these mechanisms. Emerging evidence points to the expression of VGCCs during early stages of embryonic, and in accordance with aspects of the present invention, this allows
20 ChR2 to recruit Ca²⁺ dependent cellular processes not only via its own light-activated Ca²⁺ flux but also by activating native VGCCs as differentiating cells mature. According to other aspects, lineages arising from ESCs also are to be modulated by Ca²⁺, including cardiac cells and others reporting on enhancement of hematoendothelial differentiation upon chronic depolarization of human ESCs). In all of these cases, as we observed with
25 the RA gating of optogenetic modulation, depolarization or Ca²⁺ influx is a function of other patterning and lineage-specific differentiation factors.

Recent studies have shown the induction of pluripotent stem cells (iPS) from somatic cell, significantly expanding the possible sources of stem cells in regenerative medicine but further highlighting the ongoing need for selective and highly sensitive stem
30 cell differentiation and control tools. Globally applied stimuli such as growth factors and organic compounds will affect all cells present, including non-dividing constituents of the stem cell niche as well as the stem cells and their progeny, but it is unlikely that these growth factors will have the same desired effect in all of the very different cells present in

the typical differentiation milieu. By targeting optical control to either the proliferating cells or to niche constituents like astrocytes, optogenetic control of intracellular signaling will allow selective control of the desired cell type.

Indeed, this optical specificity principle extends to the selective control of fully differentiated stem cell progeny in situ. Minimally invasive fiberoptic strategies have brought optogenetics to the fully intact, behaving mammal. Transplanted cells may require electrical activity to drive the final stages of phenotype consolidation and to fully integrate into host neural circuitry, representing the central goal of stem cell based regeneration medicine.

Compared to conventional electric stimulation or drugs, the genetic targeting of ChR2 makes it possible to specifically and reversibly drive precise amounts of activity in the transplanted ESCs and their progeny, which moreover do not require addition of chemical cofactors in vivo for ChR2 function. Finally, optically driving only the transplanted cells, with behavioral readouts or non-invasive imaging readout modalities like fMRI (and without the serious problem of signal interference from metal electrodes), opens the door to imaging and tuning the specific contribution of transplanted cells in the restoration of network activity and circuit dynamics, for example in Parkinson's disease. With these approaches and others, optogenetic technologies are applicable as valuable tools in stem cell biology and regenerative medicine.

EXPERIMENTAL METHODS

Mouse embryonic stem cell culturing

Mouse embryonic stem cells (CRL-1934, ATCC, Manassas, USA) were grown in DMEM medium (ATCC) containing medium conditioned by feeder cells (CRL- 1503, ATCC), 15% fetal calf serum (Gibco), 15ng/ml leukemia inhibitory factor (LIF; Sigma-Aldrich), 0.1mM 2-mercaptoethanol (Sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich). The cells were cultured in 75 cm² cell culture flasks (Falcon) with 20 ml medium at 37°C and 5% CO₂ and passaged every 3 days. Only undifferentiated cells in suspension were used for the experiments. After washing in phosphate-buffered saline (PBS) (Gibco, Invitrogen), cells were counted in a Neubauer counting chamber. The viability was determined by staining with trypan blue solution (0.4%; Sigma-Aldrich).

Transduction of ESCs with ChR2

Lentiviruses carrying the ChR2-EYFP fusion gene under the control of the EF-1-alpha promoter were generated as previously described. Viruses were concentrated via ultracentrifugation and redissolved in PBS at 1/1000 of the original volume. The concentrated viruses were then incubated with ESCs for 24hr and transduction efficiency evaluated using fluorescent microscopy one week after transduction. To obtain a highly and homogenously expressing ChR2-ESC colony, cells were sorted using FACS; a subpopulation consisting of the top 5% of YFP-expressing cells was collected.

Neuronal differentiation of embryonic stem cells

Neuronal differentiation was performed as previously described, with modifications. ESCs were plated on matrigel-coated dishes in embryoid body stage in complete ESC medium (see above). 24 hours later, medium was changed to ESC medium lacking LIF and including 5μM retinoic acid, and changed every second day for 5 days. As a second differentiation step, cells were incubated with neural expansion medium for 7 days consisting of N2 supplement, SHH (50ng/ml), FGF-8b (100ng/ml), bFGF (10ng/ml) and ascorbic acid (200μM, Sigma) in DMEM/F12 and changed every two days. Thereafter cells were cultured in N2 and ascorbic acid in DMEM/F12.

Immunohistochemical staining of cultured cells

Cells were fixed with 4% paraformaldehyde in PBS for 30min at room temperature. Fixation was stopped by washing cells three times with 0.1M glycine/PBS. Cells were permeabilized and blocked (4% BSA/0.4% saponin/PBS) for 30min and incubated in primary antibody solution at 4°C overnight. Cells were washed 4 times and incubated with secondary antibody at room temperature for 2hr. Cells were washed 3x with PBS, and at the final washing step DAPI was added (1:50,000). Coverslips were mounted using anti-bleaching Fluoromount. Primary antibodies were mouse anti-SSEA1 (Chemicon 1:300), mouse anti-nestin (Chemicon 1:200), chicken anti-β-tubulin (Chemicon 1:200), mouse anti MAP2ab (Sigma 1:500), rabbit anti vGlut 2 (Chemicon 1:200), and rabbit anti-a1C, -a1D, -a1G, and -a1H (all Alomone labs; 1:200). Cy3 or Cy5 conjugated donkey anti mouse, chicken and rabbit secondary antibodies (Jackson) were all used at 1:200.

RT-PCR

Cells were homogenized by Homogenizer (Invitrogen). RNA isolation was performed using Micro-to-Midi Total RNA Purification System (Invitrogen). Prior to RT-PCR, RNA samples were pretreated with DNaseI (Invitrogen) and reverse transcription conducted per manufacturer's protocol. Negative controls without reverse transcriptase did not result in amplified sequences. Mouse hippocampal total RNA was purchased from Clontech and the resulting cDNA served as a positive control. For PCR analysis, primers targeted to coding regions of two subunits each from both the L- and T-type VGCC families were used, as follows: L-type $\alpha 1C$ Forward: GTGGTTAGCGTGTCCCTCAT Reverse: GTGGAGACGGTGAAGAGAGC; L-type $\alpha 1D$ F: AATGGCACGGAATGTAGGAG R: GACGAAAAATGAGCCAAGGA; T-type $\alpha 1G$ F: CTGAGCGGATCTTCCTAACG R: TGAAAAAGGCACAGCAGATG; T-type $\alpha 1H$ F: TGGGAACGTGCTTCTTCTCT R: TGGGCATCCATGACGTAGTA; Housekeeping gene (Actin) F: GGCATTGTGATGGACTCCGG R: TGCCACAGGATTCCATACCC. 293 FT kidney cells did not express these channel subunits, as expected (Figure 8a), and PCR products of actin and L-type and T-type subunits were cloned and sequenced to confirm identity.

Long-term optical stimulation of ESCs

Key components of the hardware interface include (a) Oasis4i Controller (Objective Imaging) (hardware for x-y-z 3-axis and focus control) (http://www.objectiveimaging.com/Download/OI_Download.htm - software development kit (SDK) for the Oasis4i Controller), (b) DG4 Ultra High Speed wavelength switcher (Sutter), (c) Retiga SRV Camera (Qimaging), and (d) Leica DM6000 Microscope controlled by AHM (Abstract Hardware Model) controller. The parallel port is controlled using DLPORTIO library file (www.driverlinx.com/DownLoad/DIPortIO.htm - DLLs to for parallel port control) and camera parameters (gain, exposure) set using QCam SDK (Ver. 5.1.1.14) (<http://www.qimaging.com/support/downloads/> - SDK to control the Retiga SRV/ Exi Cameras). The custom software user interface to the optogenetic stimulation setup was developed using the Microsoft Foundation Library (MFC; Ver. 8.0) and is available on request. Briefly, regions of interest (for example, an embryoid body or a small well in a multiwell plate) to be stimulated and/or imaged are selected using the Oasis4i Controller, and their locations saved using the MFC interface. Stimulation

parameters (excitation filter wavelength, the duration of the excitatory pulse, and the frequency and duty cycle of excitation) are then set in the custom GUI. To allow stimulation space to be mapped, each region of interest can be readily programmed to receive a different stimulus pattern to operate over the many days of stimulation and
5 imaging. Similarly, imaging parameters can also be varied for selected regions, including number of images per region and exposure, gain, excitation and emission filters.

Undifferentiated cells were seeded on matrigel (BD) coated coverslips in 24-well plates in complete ESC medium at a density of 100,000 cells/well. Both native ESCs and ChR2-expressing ESCs were used in different wells on the same plate. 24 hours after
10 seeding, medium was changed to the various experimental conditions including complete ESC medium, ESC medium lacking both LIF and conditioned media from feeder cells (differentiation medium), differentiation medium with 1 μ M retinoic acid (RA) (Sigma), and differentiation medium with 2.5 μ M RA. Optical stimulation was conducted using the previously-described tools (FIG. 4). Up to 30 regions of interest (ROIs) were defined
15 per well, ensuring that all cell-containing regions on the coverslip were stimulated. ROIs were illuminated every hour around the clock over 5 days with blue light (470 nm) pulsing at 15 Hz for 10s, using a 10x objective (NA 0.3). Every 8 hours, a photomicrograph was programmed to be taken of the selected ROIs. At the end of the experiment, coverslips were removed from the plates and immediately fixed with
20 paraformaldehyde and stained as described above. Mounted slides were labeled with coded numbers by a colleague so that the investigators conducting confocal analysis were blind to treatment condition.

Confocal microscopy and image analysis

25 Confocal imaging was conducted using the Leica SP2 confocal microscope and a 40x oil objective (NA 0.75). For DAPI excitation, a 402 nm diode laser was used; Cy5-nestin was excited using a 633nm HeNe laser. 6 ROIs were randomly and blindly selected for analysis per coverslip, and 1024x1024 8-bit confocal images were obtained. For each ROI, a z-stack with 8-12 x-y-sections and a z step size of 0.98 μ m were
30 collected, thereby including all cells present in the ROI. Data analysis was conducted using ImageJ (NIH, USA) software, and after unblinding, confocal images of all ROIs of all coverslips of each condition (*e.g.*, ChR2-ESCs, optically stimulated, 2.5 μ M RA) were converted into a single z-stack. Fluorescence intensity histograms were calculated for

DAPI and nestin channels. DAPI histograms reflecting the cell numbers allowed for a normalization of nestin histograms. All nestin voxel numbers have been divided by this DAPI factor. Statistical analysis was conducted using SPSS (Chicago, USA) software. To statistically compare histograms, the parameter-free Kolmogorov-Smirnov test was
5 employed, and to compare means, statistical significance was calculated using the t-test.

Stereotactic cell transplantation

Rats (male Wistars, 250-350 g) were the subjects of these experiments. Animal husbandry and all aspects of experimental manipulation of our animals were in strict
10 accord with guidelines from the National Institute of Health and approved by members of the Stanford Institutional Animal Care and Use Committee. Rats were anaesthetized by i.p. injection (90 mg ketamine and 5 mg xylazine per kg of rat body weight). For cell transplantation, a 1 mm craniotomy was drilled over motor cortex. 1 μ L of ESCs expressing ChR2-EYFP fusion protein at a density of 50k cells/ μ L, suspended in PBS
15 were injected (26g Hamilton Syringe) into rat motor cortex (AP + 1.5 mm, ML + 1.5 mm, DV + 1.5 mm). The injection duration was 10 min; an additional 10 min delay followed before syringe withdrawal, and electrophysiology was conducted after 1 week.

Electrophysiology

20 For acute slice electrophysiological experiments, 1 week post cell transplantation, 250 μ m cortical slices were prepared in ice-cold cutting buffer (64 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 120 mM sucrose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 0.5 mM CaCl₂ and 7 mM MgCl₂, equilibrated with 95% O₂/5% CO₂) using a vibratome (VT 1000 S; Leica). After a recovery period of 30 min in cutting buffer at 32-35 °C, slices were
25 gently removed to a recording chamber mounted on an upright microscope (DM LFSA, Leica) and continuously perfused at a rate of 3-5 ml/min with carbonated ACSF (124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgCl₂, 10 mM Glucose), ventilated with 95% O₂/5% CO₂. ChR2-YFP-ESCs were identified on an upright fluorescence microscope (DM LFSA, Leica) with a 20X, 0.5 NA water
30 immersion objective and a YFP filter set. Images were recorded with a CCD camera (Retiga Exi, Qimaging) by Qimaging software. Electrophysiological recordings in cultured ChR2-YFP ESCs were performed as previously described, in Tyrode solution containing (in mM) NaCl 125, KCl 2, CaCl₂ 3, MgCl₂ 1, glucose 30 and HEPES 25 (pH

7.3 with NaOH). Membrane currents were measured with the patch-clamp technique in whole-cell mode using Axon Multiclamp 700B (Axon Instruments) amplifiers. Pipette solution consisted of (in mM): 97 potassium gluconate, 38 KCl, 6 NaCl, 0.35 sodium ATP, 4 magnesium ATP, 0.35 EGTA, 7 phosphocreatine and 20 HEPES (pH 7.25 with
5 KOH). Pipette resistance was 4-8 MΩ. Membrane potential was noted at the time of establishing the whole cell configuration. We employed pClamp 9 acquisition software (Axon Instruments), a DG-4 high-speed optical switch with 300 W xenon lamp (Sutter Instruments) and a GFP filter set (excitation filter HQ470/40x, dichroic Q495LP; Chroma) to deliver blue light for ChR2 activation. Through a 20x objective lens, power
10 density of the blue light was 8-12 mW/mm², measured by power meter (Newport). All experiments were performed at room temperature (22-24 °C).

The various embodiments described above are provided by way of illustration only and should not be construed to limit the invention. Based on the above discussion and illustrations, those skilled in the art will readily recognize that various modifications
15 and changes may be made to the present invention without strictly following the exemplary embodiments and applications illustrated and described herein. Such modifications and changes do not depart from the true spirit and scope of the present invention, which is set forth in the following claims.

What is claimed is:

1. A method for facilitating cellular development in a biological environment having a selected type of stem cell that can be distinguished from other cell types, the method comprising the steps of:

introducing a microbial opsin into a region of the biological environment that includes a selected type of stem cell, by expressing the microbial opsin in the stem cell;

introducing a light source near the stem cell; and

controllably activating the light source to direct pulses of illumination from the light source to the selected type of stem cell, for selectively controlling its growth and development independent of the growth and development of the other types of cells.

2. The method of claim 1, wherein the selected type of stem cell is as an integral part of the biological environment which includes a mixed set of cell types.

3. The method of claim 1, further including using intrinsic properties of axons and dendrites to facilitate the controlled development of young neurons.

4. The method of claim 3, wherein the intrinsic properties include one or more of: different associated chemo-attractants, temporal properties characterizing the speed at which axons and dendrites grow, and physical dimensions of axons relative to dendrites.

5. The method of claim 1, wherein the selected type of stem cell is as an integral part of tissue which includes a mixed set of cell types including cells that are targeted for control and cells that are not targeted for control.

6. The method of claim 5, further including facilitating discrete communication within the mixed set of cell types, whereby cells of individual types and individual roles in tissue development are governed.

7. The method of claim 1, further including facilitating cellular growth of the stem cell within a predetermined spatial configuration.

8. The method of claim 1, further including facilitating growth of the stem cell within a predetermined geometric configuration.
9. The method of claim 8, further including facilitating control for internal pacing of a portion of a brain, whether hypoactive or hyperactive portion of the brain being internally paced, while using another portion of the brain to facilitate control.
10. The method of claim 1, wherein the stem cells are immature cells.
11. The method of claim 1, wherein the biological environment is a tissue.
12. The method of claim 1, wherein the biological environment is a cell culture.

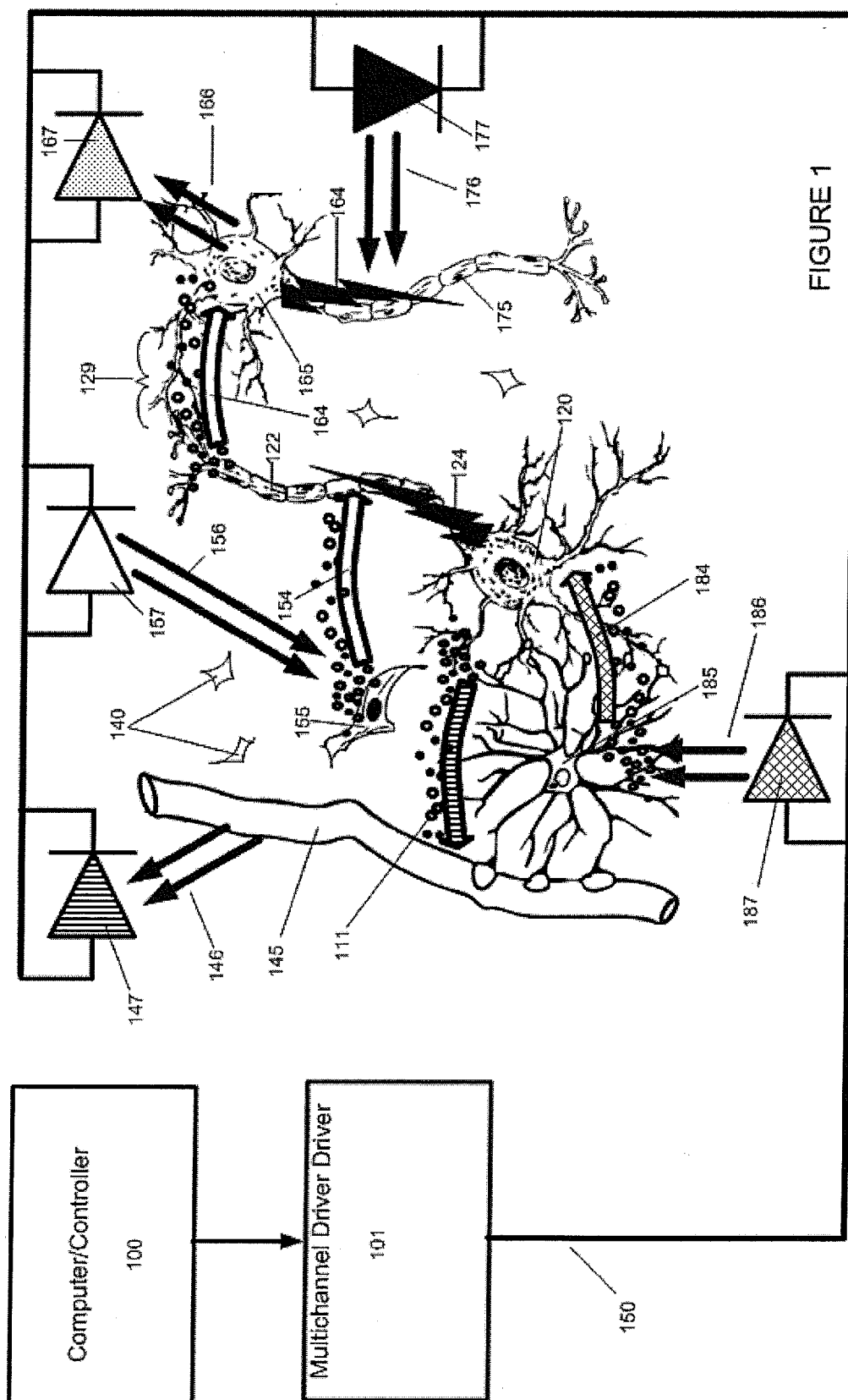


FIGURE 1

2/11

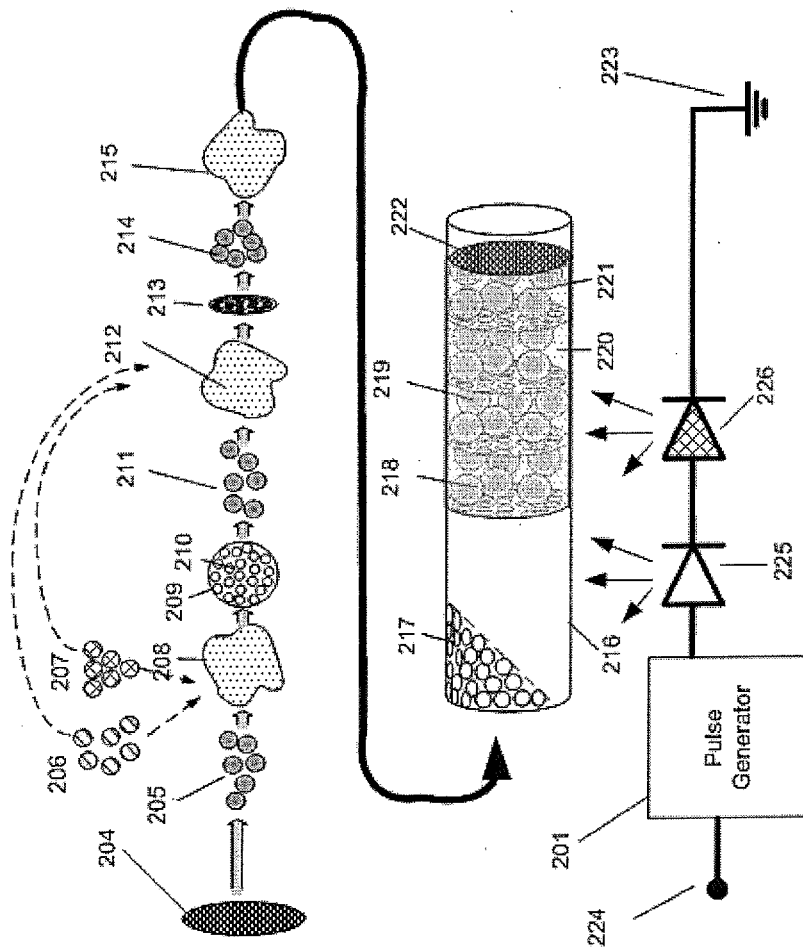


FIGURE 2A

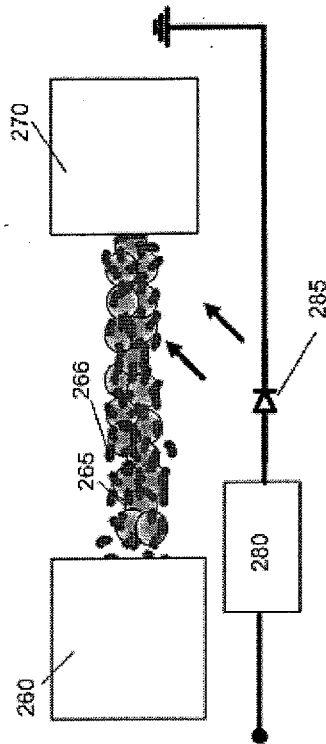


FIGURE 2B

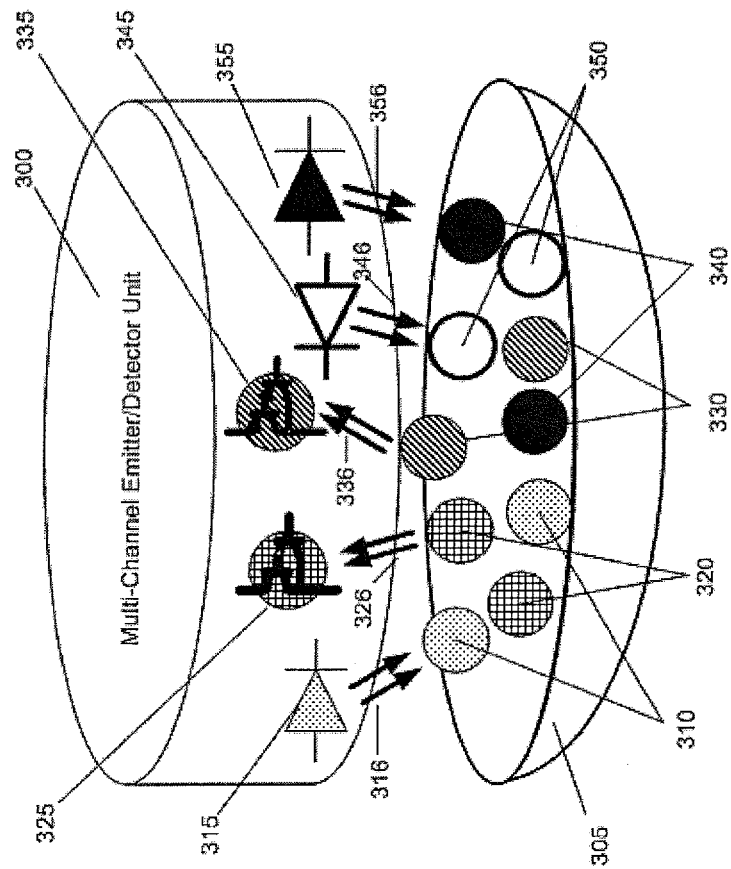


FIGURE 3A

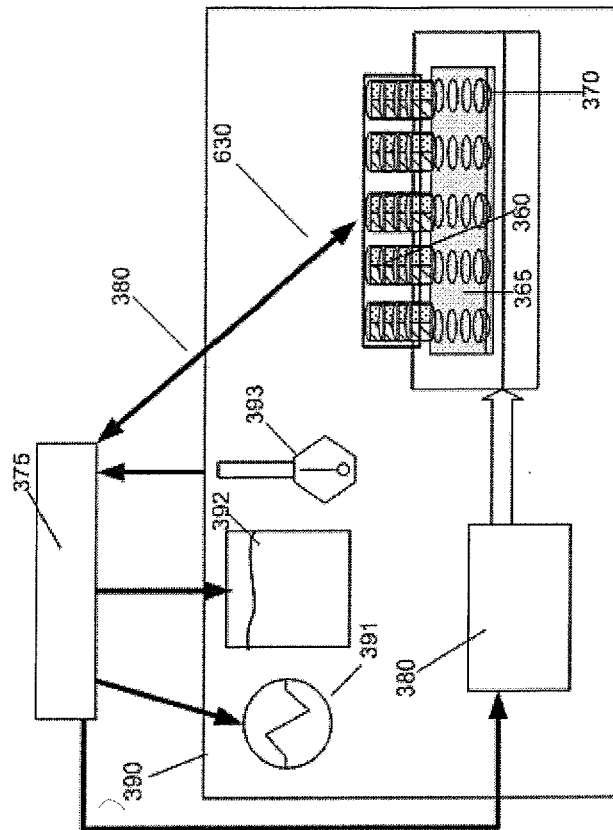


FIGURE 3B

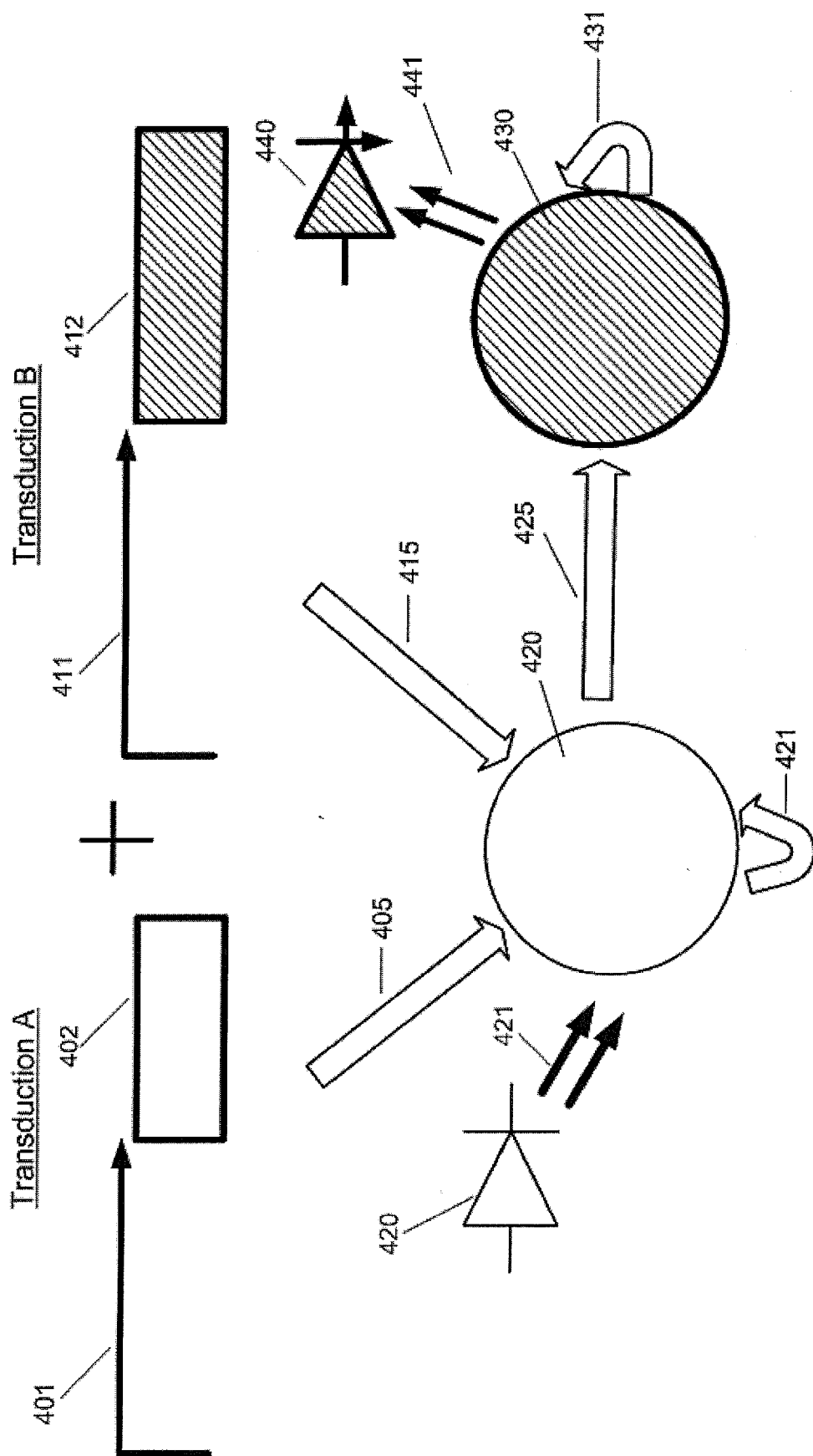


FIGURE 4

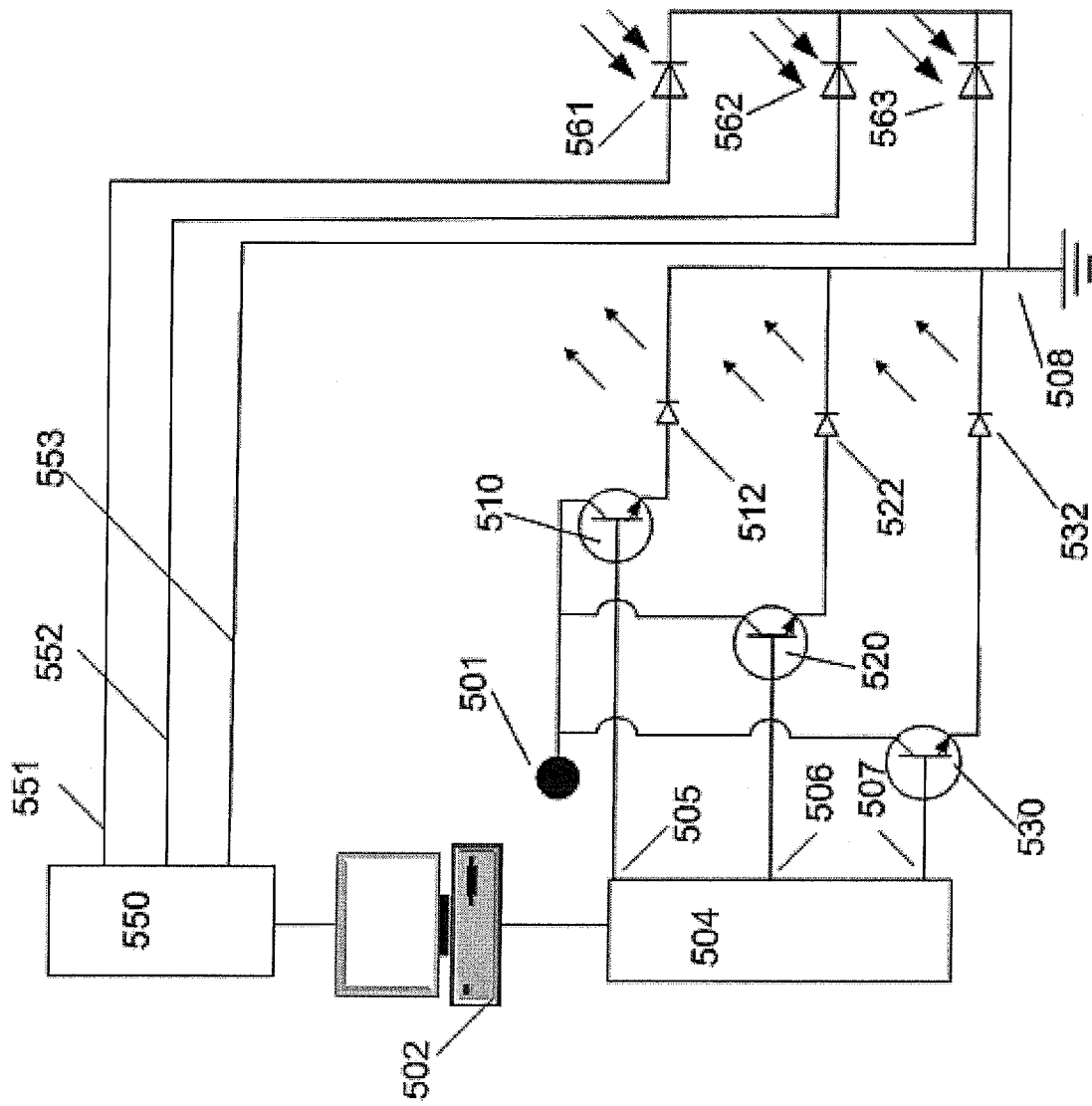


FIGURE 5

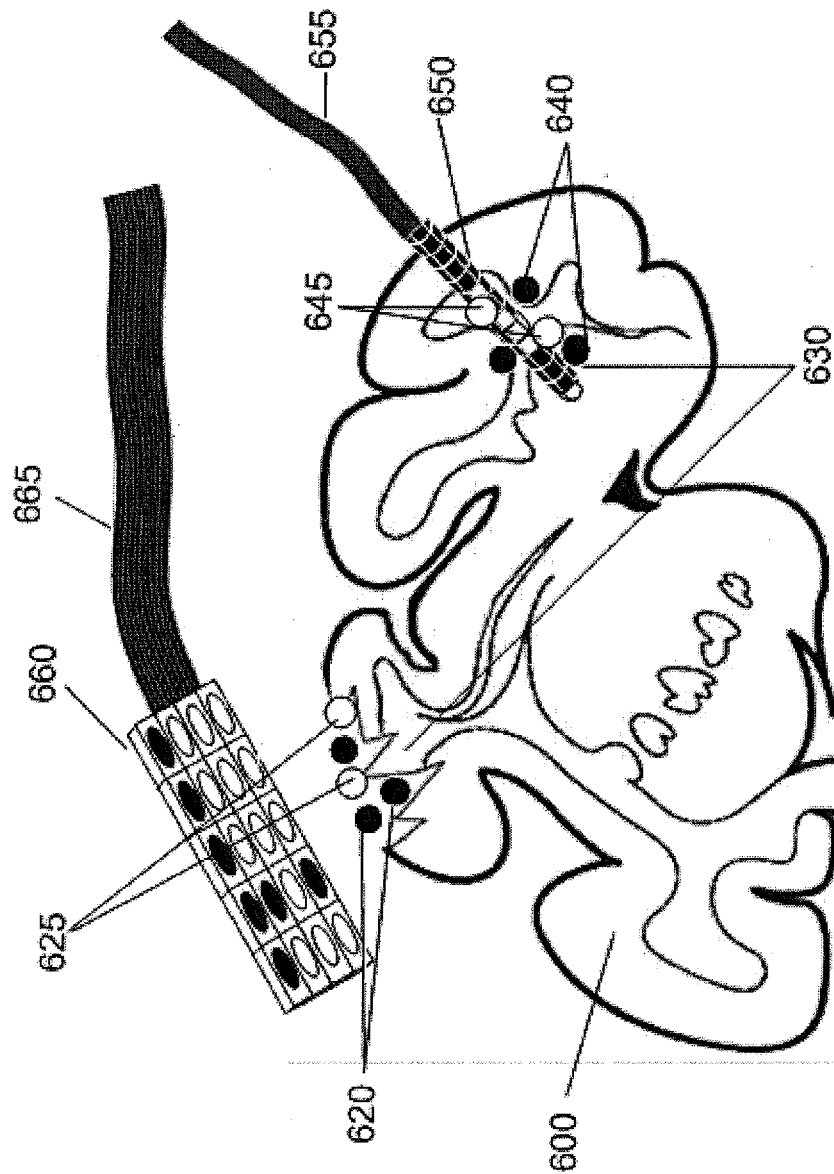


FIGURE 6

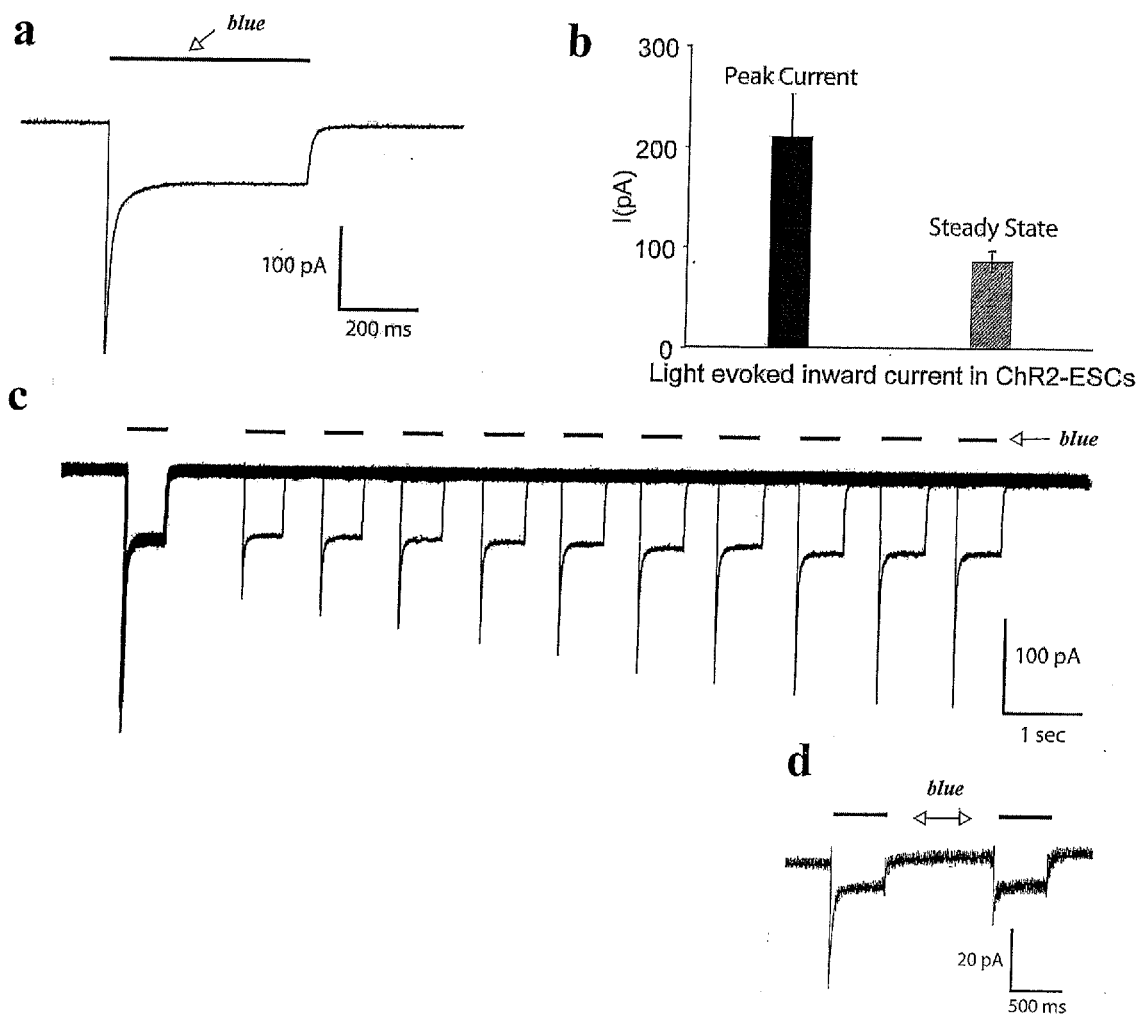


FIGURE 7

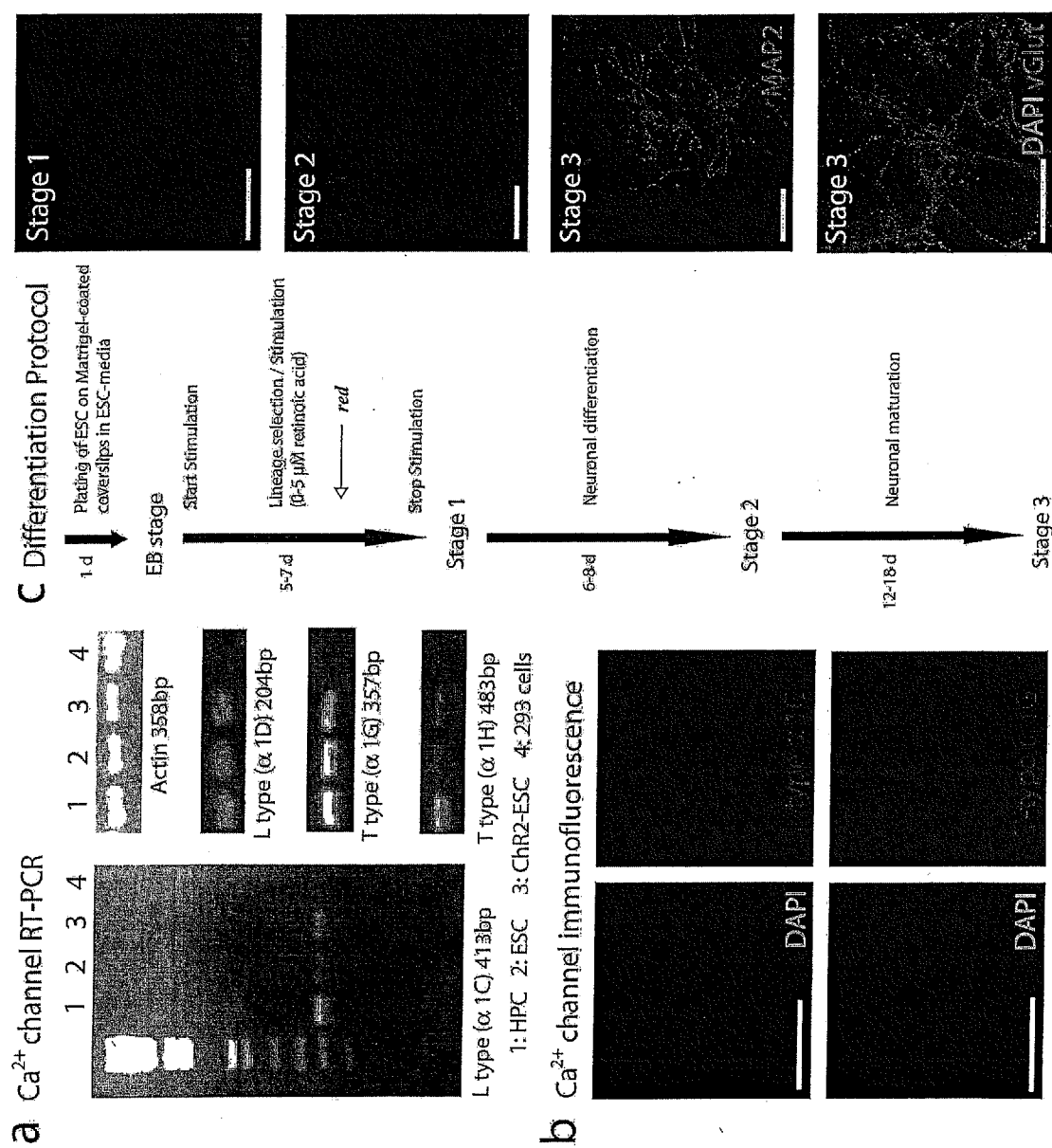


FIGURE 8

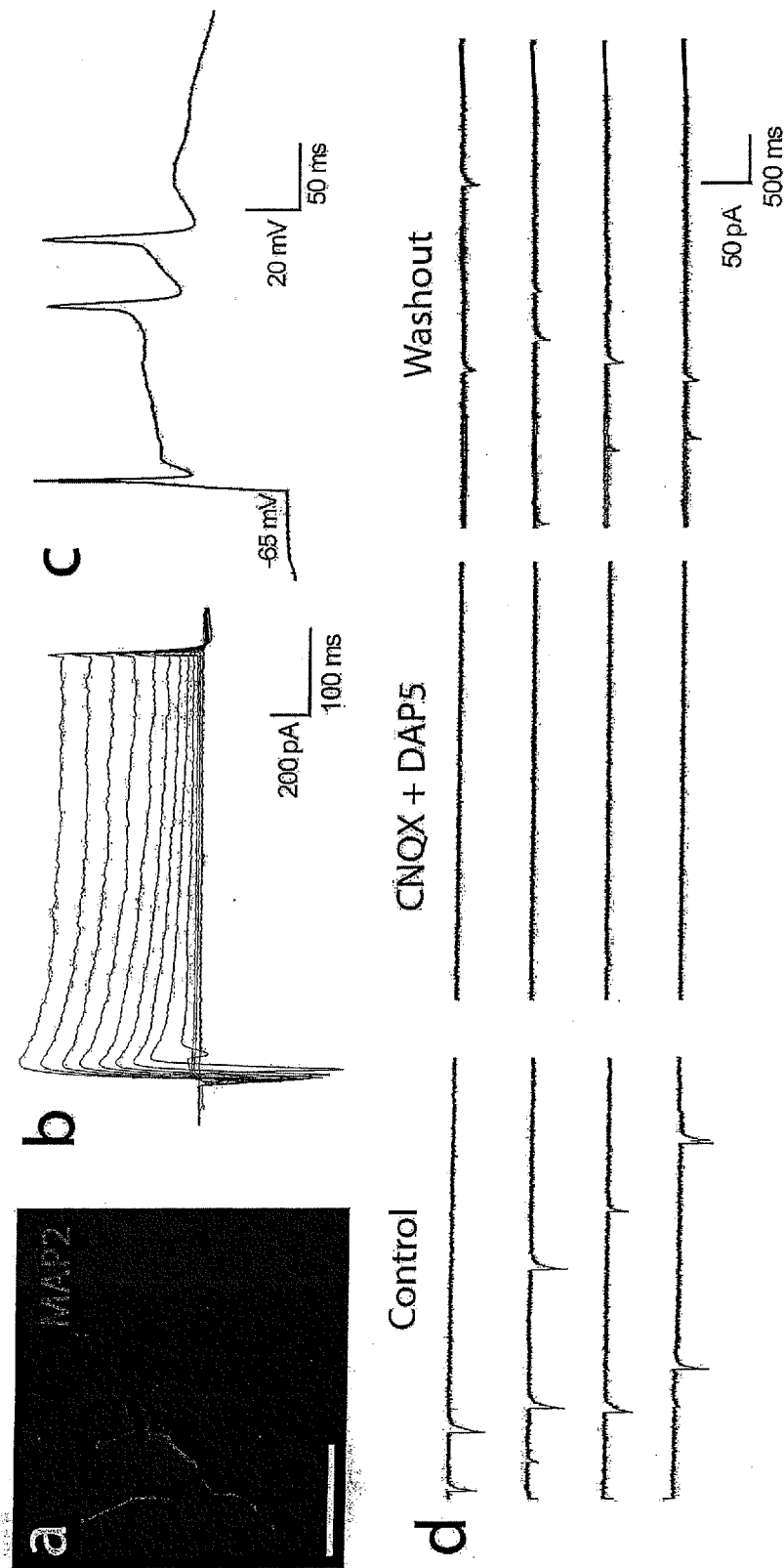


FIGURE 9

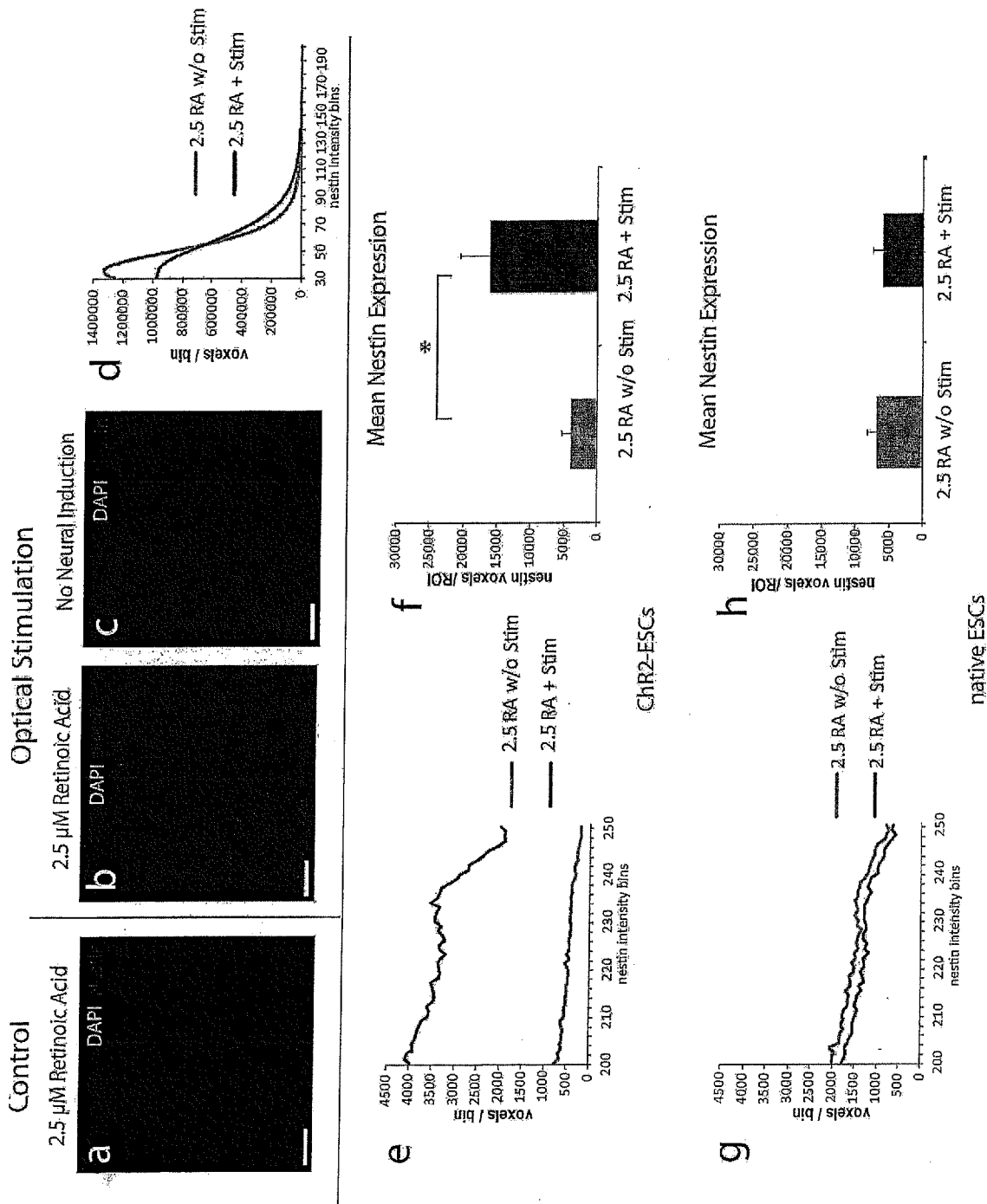


FIGURE 11