(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2012 (26.04.2012)

(51) International Patent Classification:
CI2N 5/00 (2006.01)

(21) International Application Number:
PCT/GB2011/001518

(22) International Filing Date:
21 October 2011 (21.10.2011)

(25) Filing Language:
English

(26) Publication Language:
English

(30) Priority Data:
1017820.0 21 October 2010 (21.10.2010) GB

(71) Applicants (for all designated States except US):
KING’S COLLEGE LONDON [GB/GB]; Strand, London WC2R 2LS (GB).
IMPERIAL INNOVATIONS LIMITED [GB/GB]; Level 12, Electrical and Electronic Building, Imperial College London, London SW7 2AZ (GB).
UCL BUSINESS PLC [GB/GB]; The Network Building, 97 Tottenham Court Road, University College London, London WC1E 4TP (GB).

(72) Inventors:

(75) Inventors/ Applicants (for US only): DEGENAAR, Patrick [GB/GB]; School of Electrical, Electronic and Computer Engineering, Newcastle University, Merz Court, Newcastle upon Tyne NE1 7RU (GB).
LIEBERAM, Ivo [GB/GB]; King's College London, New Hunt's House, Guy's Hospital Campus, London SE1 1UL (GB).
GREENSMITH, Linda [GB/GB]; UCL Business PLC, The Network Building, 97 Tottenham Court Road, University College London, London WC1E 4TP (GB).

(74) Agents: RICHARDS, William et al; BRYER, SHELEY, JOHN AMOR, GREENWOOD LLP, 7, Gay Street, Bath BA1 2PH (GB).


Published:
— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: CELLS AND DEVICES

Figure 1

(57) Abstract: The invention related to an in vitro population of cells comprising (i) a motor neuron comprising a transgene; said transgene encoding a photosensitive protein capable of triggering or suppressing an action potential upon illumination with light of cognate wavelength; and (ii) an astrocyte. The invention also provides an implantable device comprising a population of cells as described above, wherein said device is structured to retain the cell bodies within the device.
CELLS AND DEVICES

Background to the invention

Motor neurons transmit motor commands originating in higher brain centres to muscles and represent the output layer of the central nervous system (CNS). In amniotes, including humans, motor neurons are organized in motor columns along the rostro-caudal extend of the spinal cord. Each motor column possesses a unique identity and projects axons to a specific set of muscles.

Respiration represents one of the most basic and crucial types of motor output. The breathing rhythm itself is generated by a network of nuclei in the brainstem that form the respiratory pattern generator and then relayed to motor neurons of the 'hypaxial motor column' (HMC) in the cervical and thoracic spinal cord. There are two principal subsets of respiratory motor neurons: i) Cervical phrenic neurons connect to the diaphragm muscle, which forms the caudal boundary of the thoracic cavity and contracts during inspiration. ii) Thoracic HMC neurons that fall into two functional subgroups: motor neurons innervating the external intercostal muscle are inspiratory, whereas motor neurons projecting to internal intercostal muscle and some abdominal muscles are expiratory. Degeneration of respiratory motor neurons, and the phrenic nucleus in particular, is the primary cause of death in most patients suffering from Amyotrophic Lateral Sclerosis (ALS). It is a problem that regeneration of these neurons remains a medical challenge.

During embryogenesis, spinal motor neurons are specified from neural progenitors under the influence of at least two morphogens, retinoic acid and sonic hedgehog, that act along the rostro-caudal and the dorsal-ventral axis, respectively. Remarkably, the same two factors act together to direct the differentiation of neural progenitors derived directly from mouse embryonic stem cells (ESCs) toward the spinal motor neuron lineage. These ESC-derived motor neurons display molecular and electrical characteristic of primary motor neurons, survive when grafted into embryonic and adult hosts and are capable of innervating muscle. The same differentiation technique can be applied to human ESCs and 'induced pluripotent stem cells' (iPSCs), but the speed of motor neuron development is several-fold slower due to slower pace of embryogenesis in humans compared to mice. This technology has been extensively used to study molecular disease pathways of ALS in cell culture, but in vivo applications are so far limited to embryology, because ESC-derived motor neurons do not readily integrate into host neural circuits when grafted into the adult central nervous system.
One of the reasons for this limitation is the fact that the adult central nervous system expresses numerous axonal repellents, which are often associated with the myelin-sheath. These repellents largely prevent the outgrowth of axons from ESC-derived motor neuron grafts and lead to a failure to reinnervate muscle effectively. Yohn et al. (Yohn DC, Miles GB, Rafuse VF, Brownstone RM. Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. J Neurosci. 28:12409-12418) recently reported an alternative experimental approach, in which the authors grafted ESC-derived motor neurons into a peripheral hindlimb motor nerve, rather than the CNS. The grafted cells survive in their ectopic peripheral location and innervate the muscle at the end of the nerve.

In neurons, communication signals are transmitted via electrical impulses known as action potentials. Normally the neuron is negatively charged, but during an action potential pulse, the negative polarisation is temporarily negated. These action potentials are generated by the opening and closing of voltage-gated sodium and potassium ion channels. The membrane voltage of a neuron can be also transiently depolarized by means of extracellular electrodes to induce an electrical impulse. Presently, neuroprosthetists generally use electrical stimuli to induce action potentials in neurons. However, neuroelectronic interfaces suffer from many fundamental drawbacks which can be summarised as follows:

Inhibition: Electrical stimuli can excite but not inhibit action potential firing.

Cellular targeting: It is difficult to confine electrical fields to target specific cell types or structures.

Scalability: Large arrays of electrodes require smaller electrodes which have charge density problems leading to degradation.

Power dissipation: In neural tissue, power dissipation becomes a problem for large arrays of stimulators.

Biocompatibility: Electrodes can degrade releasing cytotoxic components, or if overstimulated, can release free radicals due to electrolysis of water, which can also be toxic to the surrounding cells.

An alternative to electrical stimulation is the use of light to generate action potentials in neurons. The photo-stimulation concept was first demonstrated in 1971 by Richard Fork who used a high power laser to stimulate action potentials in the abdominal ganglion of the marine mollus Aplysia California. Since then scientists have exploited developments in nanotechnology and genomics to photosensitize cells. These sensitization methods can be divided into the following categories of useful techniques:
A first category is photolysis of caged neurotransmitters, which was also the first modern photo-stimulation technique. Neurotransmitters, rendered inactive with covalently bonded blocking ligands are released into the solution around the neurons. The blocking moiety bonds with the neurotransmitters are then broken with the use of UV light. The result is a great localized increase in the neurotransmitter concentration in the vicinity of the light spot, which can excite a neuron cell. Due to its wide expression in the central nervous system (CNS) neurons, caged-glutamate is the most commonly used caged neurotransmitter. Such uncaging is however not practical in in-vivo application due to the UV light requirement and the constant perfusion of caged molecules.

A second category involves the incorporation of photoswitch-linked ion channels on the cell membrane. These can come in the form of modified ion-channels with light gated opening mechanisms. The modification method can be fully genetic, fully chemical, or a mix of the two. Existing ion channels and receptors can be genetically modified to receive photo-switchable arms which can act to open or close the active site. These can include hyperpolarizing (potassium) shaker channels and/or depolarizing modified glutamate channels. It is thus conceivable that embryonic cell culture at the point of differentiation or post differentiation such channels could be modified to induce continued photosensitivity, before implantation to a target destination. However, there may be problems with the long term stability of such channels with this approach.

A third category is the genetic incorporation of inherently photosensitive ion channels. Channelrhodopsin [Nagel, G; Szeilas, T; Huhn, W, et al. "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel" PNAS Vol: 100 (24) ppl 3940-13945, Nov 2003] and Melanopsin [Melyan, Z; Tarttelin, EE; Bellingham, J; Lucas, RJ; Hankins, MW "Addition of human melanopsin renders mammalian cells photosensitive" Nature 433 (7027): 741-745 Feb 17 2005] have been described. The former is a photosensitive ion channel which is opened on absorption of a photon and transports depolarising sodium into the cell. The latter is a G-protein linked cascade which inherently exists in the human visual system. In addition, complementary light activated chloride pumps such as halorhodopsin have been discovered which can hyperpolarise i.e. deactivate neuron firing [Zhang, F; Wang, LP; Brauner, M, et al. "Multimodal fast optical interrogation of neural circuitry" Nature Vol 446 (7136) pp633 April 2007]. To date channelrhodopsin has been successfully used to stimulate the nervous system of numerous animal models including primates [Han, X; Qian, XF; Bernstein, JG, et al."Millisecond-Timescale Optical Control of Neural Dynamics in the Nonhuman Primate Brain" Neuron Volume: 62 (2), pp91-198, 2009].
Considerable effort is now being made to develop optogenetic stimulation methods for retinal prosthesis, and for deep brain stimulation. Such optogenetic methods have significant benefit over current electrical and electro-optical approaches as described above. However, for neurodegenerative disorders whereby there is physical or degenerative damage to the nerve fibre, there is simply no longer anything to stimulate. Furthermore, there may be ethical and safety considerations in using viruses to express non-native proteins in human beings.

The present invention seeks to overcome problem (s) associated with the prior art.

Summary of the invention

This invention describes populations of light sensitised motor neurones, and devices comprising same. These motor neurones can be triggered to fire (or inhibited from firing) by light. These cells can be produced from embryonic stem cells or induced pluripotent stem cells derived from the recipient's own cells. Therefore the invention provides a new mode of repairing or augmenting a subject's nervous system, and provides a new way of tissue matching those neurons avoiding problems associated with direct gene therapy applications and existing electrical stimulation/neural repair techniques. The invention therefore is of particular use in those diseases such as motor neurone disease, where there is an atrophy of the existing neural circuitry and/or communication system. This invention is based on both the biological and technical advances made by the inventors.

In one aspect the invention provides a population of cells comprising

(i) a motor neuron comprising a transgene; said transgene encoding a photosensitive protein capable of triggering or suppressing an action potential upon illumination with light of cognate wavelength; and

(ii) a companion cell.

Companion cells are required for the maintenance of motor neurons. The companion cell may be an astrocyte or an oligodendrocyte or other suitable cell capable of supporting motor neurons. Most suitably the companion cell is an astrocyte.

Thus in one aspect the invention provides a population of cells comprising

(i) a motor neuron comprising a transgene; said transgene encoding a photosensitive protein capable of triggering or suppressing an action potential upon illumination with light of cognate wavelength; and

(ii) an astrocyte.
Suitably said population of cells is an in-vitro population of cells.

Suitably the motor neuron and astrocyte are each derived from stem cell(s). Stem cells may be embryonic stem cells (ESCs) or pluripotent stem cells such as induced pluripotent stem cells (iPS/iPSCs).

Suitably embryonic stem cells are from culture so that their provision does not involve the destruction of embryos, more suitably does not involve the destruction of human embryos. Suitably the stem cells are induced pluripotent stem cells (iPS/iPSCs) which have the advantage of omitting any involvement of embryonic stem cells.

Suitably the photosensitive protein comprises channeirhodopsin (ChR) or meianopsin or halorhodopsin (NpHR) or a variant thereof; more suitably the photosensitive protein comprises channeirhodopsin (ChR) or meianopsin or halorhodopsin (NpHR).

Suitably the photosensitive protein comprises channeirhodopsin (ChR) or meianopsin and stimulation of said photosensitive protein triggers depolarisation of the motor neuron.

Suitably the photosensitive protein comprises halorhodopsin (NpHR) and stimulation of said photosensitive protein triggers hyperpolarisation of the motor neuron.

Suitably said population further comprises an oligodendrocyte.

Suitably said population comprises 50 to 5000 motor neurons.

Suitably said population comprises 500 to 50,000 astrocytes.

Suitably said population comprises motor neurons and astrocytes in the ratio 1:10.

Suitably said cell(s) further comprise a second transgene encoding a genetic marker. This is advantageous in selecting and/or preparing the cells of the invention.

In another aspect, the invention relates to a population of cells as described above for use as a medicament.

In another aspect, the invention relates to use of a population of cells as described above for the manufacture of a medicament for Amyotrophic Lateral Sclerosis (ALS).
In another aspect, the invention relates to a population of cells as described above for use in the treatment of Amyotrophic Lateral Sclerosis (ALS).

In another aspect, the invention relates to an implantable device comprising a population of cells as described above, wherein said device is structured to retain the cell bodies within the device.

Detailed Description of the Invention

The invention describes cell mixtures and devices to help pace or restore lost neural function using a mixture of genetic and stem-cell therapies and prosthesis. The concept is to create an implant consisting of specific types of neurons and glia derived in vitro from stem cells in order to innervate target tissue or send signals to other neural circuits. The implanted stem cell-derived neural cells are genetically engineered to express channelrhodopsin or other light sensitive ion channels, ion pumps such as halorhodopsin, or cascade systems such as melanopsin. This allows a light source to induce or inhibit signalling in these cells. Light is guided into (or directly illuminated on) these areas. The cells can be contained in a special enclosure which can prevent migration from the target location and act as a waveguide to ensure even illumination throughout the stem cell implant. Different wavelengths can then be used for depolarization or hyperpolarization by activating appropriate optically active ion channels or ion pumps. This in turn can ensure the initiation or inhibition of synchronous contraction in subsequent muscle.

Contrary to known artificial electrical pacemaking and neuroprosthetic technologies that are based on the application of a current or an electric field, here the differentiated implanted neurons are controlled by the opening and closing of ion channels or ion pumps with light. This operation is much closer to the normal physiology of the target neurons. Using light to trigger contractions in target muscle, can be achieved simply by pulsing the light on the communicating nerve cells. Since the light can be pulsed with high temporal resolution, the rate can be precisely controlled.

Using light instead of electricity enables also remote stimulation. For example, in the case of the eye, the optics of the eye itself can be used to target the implanted neurons. Additionally for nerves close to the surface of the skin such as the parotid nerve, it may be possible to stimulate by optical illumination via the skin. This introduces a significant reduction in the invasiveness and, potentially, in the operative complexity of pacemakers and may avoid some of the major complications of the traditional electrical approach.
An exemplary application of the invention is to the phrenic nerve. Rather than attempting to reconnect implanted motor neurons to normal spinal circuits as in prior art studies, we will implant motor neurons with an artificial input stimulated by light. The light may be provided by an optoelectronic pacemaker. We equip the SC-derived motor neurons with an excitatory photosensor transgene such as channelrhodopsin (Chr). The pacemaker device or other light source will encode an artificial respiratory rhythm in a pattern of light pulses. The encoding will suitably include both the neural firing patterns and the biophysical response of the photosensitisation agent. Transmission to the SC-motor neuron graft may be via optical fibre cables.

In the prior art, motor neurones are cultured on their own. They are swiftly lost, typically after 2-3 days of culture. They become overgrown with other cell types and the motor neurons die. Prior art attempts to overcome this problem involve culture with primary astrocytes derived from adult brain. This is clearly impossible for humans since preparation of primary astrocytes requires sacrifice of a human brain.

By contrast, we disclose the separate sorting of motor neurons and of companion cells such as astrocytes in vitro. This separate sorting is accomplished using transgene markers. Once the two cell types are prepared, we teach their combination into a population according to the present invention. This may be regarded as reaggregation. These populations are stable, and mature morphologically in culture. This provides a great advantage over the art.

Optogenetic neural stimulation

The invention makes use of genetically incorporated light sensitive functional proteins which either react directly or indirectly to the action of light. These proteins are referred to as 'photosensors' herein. Directly gated ion channels and pumps such as channelrhodopsin2 (ChR2) and halorhodopsin (NpHR) act directly on light action, i.e. an absorbed photon will open an ion channel, or cause a pumping action. ChR2 and NpHR can depolarize and hyperpolarize cells respectively. It is also conceivable that modified receptor groups such as glutamate, acetylcholine and GABA could act on light stimulation to stimulate or inhibit neuro-electric activity. Such receptors would need to incorporate light sensitive moieties such as retinal to function. In addition, amplifying cascades could be used for depolarisation and hyperpolarisation. G-Protein linked cascades such as Melanopsin are particularly applicable. Additionally it is conceivable that existing cascades in the heart such as the G-protein linked Adrenergic receptor system could be used to modulate cAMP activity and thereby cardiac activity. These light sensitization methods can be implemented via genetic
manipulation of the cells. The advantage of such genetic manipulation is that the active agents are continuously produced by the cellular machinery circumventing any decay over time. Delivery methods include chemical insertion, electroporation, lipofection, viral (e.g. Adeno Associated virus or lentivirus) transfection.

5 Definitions
The term 'comprises' (comprise, comprising) should be understood to have its normal meaning in the art, i.e. that the stated feature or group of features is included, but that the term does not exclude any other stated feature or group of features from also being present.

Genetic Markers
One or more genetic marker(s) are used to select the cells of interest. These are selectable in the sense of enabling their selection which may be by visual means such as by flow cytometry e.g. fluorescence activated cell sorting (FACS) or other similar technique.

In principle the genetic marker(s) may provide a chemical or survival based selection for example positive/negative selectable markers. Examples of these include the well established Blasticidin S (BlaS) or thymidine kinase (Tk) gene of herpes simplex virus (HSV) which markers can be selected positively by their ability to induce BlaS resistance and/or negatively on the induced sensitivity towards gancyclovir (GANC). However, suitably the markers used enable selection by visual means.

25 Of course it may be possible to directly select cells having the desired fate e.g. to directly select motor neuron cells from a pool of cells by virtue of detecting naturally occurring marker protein(s) characteristic of that cell type. For example if motor neurones express a particular cell surface marker, then those cells might be selected by applying an antibody for said naturally occurring cell surface marker and selecting those cells which display such a naturally occurring marker. This advantageously avoids the need for insertion of a genetic marker for this purpose. However, most typically the cells of the invention will be selected using a genetic marker. Thus suitably the cells of the invention comprise a genetic marker.

Suitable examples of markers such as naturally occurring surface antigens include human CD2 and/or human CD14 as surface tag(s) for motor neurons. For other cell types alternative markers are used as mentioned.
Suitably CD14 wild type sequence is used.

Markers which are (or have been mutated to be) functionally inert but still bind antibody and therefore are still suitable for use for cell sorting may be used.

For example, CD2 missing the cytoplasmic tail may be used. This can be very protease sensitive.

For example, CD14 can be used. This has the advantage of being protease resistant. CD14 has no cytoplasmic tail since it is GPI anchored.

For example, wild type marker CD14 can bind lipopolysaccharide (LPS). This binding is typically in co-operation with TLR2. CD14 mutants are known which have reduced LPS binding. CD14 mutants are known which have reduced LPS binding but which still bind anti-CD14 antibodies. An exemplary mutant is referred to as 'delta-AVEVE' and lacks beta-sheet-3 in the LPS-binding pocket as described in Viriyakosol and Kirkland (J Biol Chem. 1995 Jan 6;270(l):361-8.)

The DNA sequence of this mutant CD14 isoform (including the poly linker) is shown below:

1 GTCGACACGG GTCCACCATG GAGCAGCGGT GCTGCTGCTG CTGGCGCTGG
2 61 TGGACGTCCG TGGAGGCAGG CCAAGACTTT GTGAGCCTGA AGATGAAGAG
121 TCTGCAACTCC TCCCCGACTC GTCCAGAACG CTTGCTGATG TGTGCTATCC
181 ATGCGGGGCGG TGCTCACTTA GAGGGGCTTC TAAAGGGCCT CGATGCACGC
241 GGGATGTATGC TGACACGTACG GAGGATCTCG GGTTGGGCGG GCTCACGAGG
301 AGGGTCCTGCG TCGACACTCG GGCGGCGGCC TCCGGTGCTC AGGACTACCC
361 AATGGAGGCT CAGAGGCTCT TAAGATAAGC GCAACATGCT TCCGGCTGCT
421 CAGAGCTCGCT ACTTCCCAGC TGGCCTGACT GCCAAGCTG TGGGCGAGCA
481 GGTGGCGCAG GGTGAGCAAG TGCTCAAGCG CAGGCTCTAA GTTACGAGC
541 CACACTCGCG TGGCTTTCCT TGGGACAGG TGGGGGCTTT CCGCGGTCTT ACCAGCCTAG
601 ACGTGCGGCT CAATCTGGGA CCTGCGGCAA GCGGACTGAG GGGGCTCTCA TGGCCCAACA
661 AGTTCCCGCC CATCCAGGAT CTAGGCGCTG GCAACAGCA AATGGGACGG CCCAGCGCC
721 TGGCGGGGGG CTGGGCTGGA GCAGTGAGC AGCCACAGC CTTACAGTC AGCCACAACT
781 GTGCGGCAGC CACGTAACCC CTTAGCCGCT CAGAAGCTAC GTCGCCGAC GCCCTGAAC
841 GGTCTAAAGT GCTGGTGGCTG GGGCGGACAG AGGTCTGCTA AGGAAGCTCC GCCAGCTCA
901 GAGGTCTCAG TCTCAGCTGC AACAGACTGA ACAGGGGCGC GAGGTCGGAG GATGCCC
961 AGGTGGGATAC CTTGACACTG GACGGGAATC CTTCTGGTCT CTTGAAGAC CCCTCCCAAC
1021 AGCGAGGCGT AACGAACTCC GGGGTGGTGG CACGGTGCTG AGGCTGGGGG
1081 GGTTGGGCGG ACCCTGGTGG CTGGCTCAAG GGGGCGGGG CTTTGCTCAA ACTAGGCGG

Alternate markers suitable for use in the invention include human CD4 or human CD8. CD4 or CD8 proteins can be rendered functionally inactive by deleting their intracellular, C-terminal domain, which prevents them from interacting with signalling molecules, such as the Src-like kinase Lck. Thus these mutant forms are suitable for use in the present invention.
Suitably marker transgenes comprise a cell type specific promoter (e.g. motor neurone specific promoter such as MNX for motor neurones; e.g. astrocyte specific promoter such as GFAP for astrocytes) operably linked to a marker sequence. Typically the marker is any marker which can be used to sort the cells by expression. Suitably the marker is a cell surface marker such as a cell surface antigen (cell determinant or CD marker).

Typically sorting (selection/purification) of the cells of interest is done by magnetic sorting. Exemplary protocols are those for commercially available sorting reagents such as Miltenyi microbeads, magnetic columns and magnets.

Suitably the genetic marker comprises a transgene. Suitably said transgene comprises a promoter specific for the cell type which it is desired to select e.g. a motor neurone specific promoter or an astrocyte specific promoter or an oligodendrocyte specific promoter. Suitably the transgene further comprises a selectable marker which is placed under the control of the cell type specific promoter. In this manner, the selectable marker is only expressed when the cell type specific promoter is active i.e. the cell type specific promoter is switched on or induced. This advantageously allows identification of only those cells of the desired cell type e.g. motor neurons.

The promoter may be chosen by the skilled worker; the promoter needs to be capable of operating in the cell in which it will be placed (e.g. human cells) and needs to exhibit the appropriate cell-type-specific activation (e.g. induced in astrocytes).

Clearly the promoter will vary depending on the cell type being selected.

For motor neurons suitably the promoter comprises the Mnx1 (motor neuron and pancreas homebox 1 / Hb9) promoter. An exemplary sequence of the Mnx1 promoter is 9.0KB mouse Mnx1 promoter;

```
1 TTAATGCAAT AAAACAGACA CGCAGGCTCC CCTTAAATGG TTCTTTCATA ATGAATAAAT
61 TTAAGCAGGC TAAATAATAT ATAAACTAGC TCAATTGTCG AAGTTGATTT GTATTTTGT
121 TAAATGTTGA AGTAATTACC ATAAACTAGC TCAATTGTCG AAGTTGATTT GTATTTTGT
181 GTAATTTTAT TCCCTTCTGT GGTGAAGAAA ATTCATTTTT ATGGAAATTG GACCAAGCCT
241 ATAAGGCTCA TAAATCGTGG TGTAGGCTGC AGGCCAGAGTC TAAATGCGGA CATATTTCCAT
301 TTATGTTTTA ATAGAAATAAT TCATCTTCTG TTTTAAATGAA ATGAATTAGG AGGGGATAGG
361 GGGCTCTGGAG GGGGTGCTTG AGAAGGGCTG ATGACCTTTC TTCTCCTCAT
421 GCAAGGCCCA GGGCTAGAGG TCCCTGACT CAGTCAAAGA GTGGAGAGAG CTGAGGAGG
481 CCTGGAGGCA GGTGATTCTG TACCTGACTG AGACACCTTG CAGCCCTGAT TGGGAGGGG
541 GGGGGGGG GGCAATTCAC ACCCTGTATT CTACCCAGGC TAAAGTTTCT TCTATCTGGT
601 TCCCTCTTG TCCCAATACT GCAGAAGGG ACCCTCCTTC GCCTGAGAAT GAGTGAAGCT
661 GTTCAGCGTG CTGCAGACAA GCCCTGCTTG CATCTGCCCT AAGGGATCTGA TATCCTACAC
```
AAATGTCTT TGGCTCTCTG GGGTCAGAAAG CATCCAGCGC AGGGCTGAGC TCAGTCTCTT
CTCTATGTTC TAAGTACACA GCAAAACAAA CAAAAACCCG ...
GATGATTCGG GTCCATGAGG TCCACCCCAG GAAAATCTCA TCTGCAGCCA CCCTTTTTTT
ATTATATGTA
AAAATGTCTT
GAAATATATC
AAAATGACTG
GGGTGCATGT
TTCCAAGCAC
GGGGGGGGAC
TAATAAAGGA
CATATTCAAG
GGGAGCAGAG
GGTAGGAGAG
GTTATATTCA
TGTGGTTATT
ATAACATCTT
AGCAGTAGGG
CCACCATCAC
CCTCAGGTTG
TACTAAAACT
GGAGGTGGTT
TCCAGGCTCT
CATTCACACT
CCATTGGATT
CTCTATGTTC
GCTGATGGTA
TGGCTTCCTG
AGGAAATGAA
AGCCCACAGG
TTTCCCCTTC
CGGATGACTT
GTCGCCTCTG
GCTGGAACTGG
GAACACTGGG
ACAACCCAGG
GTCCATGAGG
AGAGCCAGCT
GTAACTTAAT
CTCATGGAGT
TCCTGAAGGG
GCTGGCAAAG
CATCAAGTCT
CCACTCGTAA
GTCTTCCTCT
GGGCTGGGTC
GGGGATAGGG
GATTAAATGT
GCAAGACCCA
AACACACCTT
CACTGAGCCA
CCATGTGGTT
TCCTCAACTA
GGAGGAAGTG
CACACACATAC
GAGAAGGGAG
TCTCAGCAGC
GACAGCAGCA
AAATGTCTT
TGGCTCTCTG
GGTCAGAAAG
CATCCAGCGC
AGGGCTGAGC
TCAGTCTCTT
CTCTATGTTC
TAAGTACACA
GCAAAACAAA
AAAAACCCG
GATGATTCGG
GTCCATGAGG
TCCACCCCAG
GAAAATCTCA
TCTGCAGCCA
CCCTTTTTTT
AAAATGTCTT
TGGCTCTCTG
GGTCAGAAAG
CATCCAGCGC
AGGGCTGAGC
TCAGTCTCTT
CTCTATGTTC
TAAGTACACA
GCAAAACAAA
AAAAACCCG
GATGATTCGG
GTCCATGAGG
TCCACCCCAG
GAAAATCTCA
TCTGCAGCCA
CCCTTTTTTT
6601 CCTCCTCTTT TCTCTTTGTT TGTTTTGTTG TTGTTTTTTT CAAGACAGGG TATCTCTGTG
6661 TAGCATTTTT GGTTGCTGTC CTGGAACTGG CTCTGTAGAC

Example the MNX promoter from the BAC RP24-351I23 may be used. This has the advantage that the extra genomic...
DNA present in this BAC (compared to the exemplary MNX1 promoter sequence given above) can produce a better effect in use, such as behaving more like a knock-in.

For astrocytes, the promoter comprises the GFAP (glial fibrillary acidic protein) promoter. An exemplary sequence of the GFAP promoter is the 2.2KB human GFAP promoter:

For oligodendrocytes suitably the promoter comprises the MBP (myelin basic protein) promoter. An exemplary sequence of the MBP promoter is the 1.4KB mouse MBP promoter:

```
1  AAGCTTTGAG AGAAAAAGGGA CCAGATCTTA TTCTCTACGG TGGCTTTAAC ACTTAGAGAA
5
6  1 AATGCAATCC CTTAATCACA TAAGTCTACG ACAGTGAGGA TATAGGAGAA GGGCATGGCG
10
14 2 TAGAGGATGT GTGCTAAGCA TACTCTGGTG CATGCGGCTG TAATAGCTTG GGGAGGTGGA
19
18 1 CAGGCTGGGG GTGAAATAGG TAGTGAGGAT ATGATCAGGT TTRGGTAAAT ATCAGTGGGT
23
22 1 GGGAGAGGG GTGTAAGGTGT GTGGAGATGT TAGAATAGTC TTCACTGGTT
27
30
31
35
5 1 AGGTTAGACG ATGATAAAATG GGTGATAGAC TGGAAAGGTT GTCAAAGAG GATAAGGAA
10
51 1 GGTGAGCTTA CGCGTATTTTC TAAAGTCACT GATAGCTGGC GGAAGAACGG TTAAGTTACA
15
56 1 TCCATTAAA CCACACAGGA AGCTGAGAGG GAATGGACCT GCTGGCGTTG GTGAGGAAAG
61
65 1 CTTTGCAATT CCGGTTGCTG TGGTTGGAAG GTTCAGTGGC CCCACTGGCA GCGTGGAGGT
70
74 1 TACTGGGGCT TACAGAGGAG CCAGATCCCA ATGCCACAGG TAAACAGTC CCGGACCCAG
80
85 1 AGGCGATCGT AAGTCCTGGG TGGCTAAAGG CCGTGGGGGT TCTTTATTTT GAGAACTTGA
90
94 1 AGGCAGGCCG CTGAGCAATG TATACCAAGG AGCTCAACAT CAGGTAGAGC CCTTCTGTTG
100
105
110
114
120
125
130
135
```


After cell sorting, endogenous (non-transgene) cell type specific markers can be assayed to verify the sorting (qualify control) if desired.

Reference Sequence

When particular amino acid residues are referred to using numeric addresses, the numbering is taken using the parent or wild type amino acid sequence as the reference sequence. This is to be used as is well understood in the art to locate the residue of interest. This is not always a strict counting exercise - attention must be paid to the context. For example, if the protein of interest such as channelrhodopsin is of a slightly different length, then location of the correct residue in the channelrhodopsin sequence corresponding to (for example) residue 128 may require the sequences to be aligned and the equivalent or corresponding residue picked, rather than simply
taking the 28th residue of the sequence of interest. This is well within the ambit of the skilled reader.

Mutation

Mutating has it normal meaning in the art and may refer to the substitution or truncation or deletion of the residue, motif or domain referred to. Mutation may be effected at the polypeptide level e.g. by synthesis of a polypeptide having the mutated sequence, or may be effected at the nucleotide level e.g. by making a nucleic acid encoding the mutated sequence, which nucleic acid may be subsequently translated to produce the mutated polypeptide. Where no amino acid is specified as the replacement amino acid for a given mutation site, suitably alanine (A) is used.

Where polypeptides of the invention such as photosensor proteins of the invention are mutated or varied, this will always be with the proviso that the appropriate function, or level of function, is retained. Guidance is given herein, and in particular in the examples section, as to how such properties may be easily tested. Clearly, any non-functional mutants tested should be discarded and would not form a part of the invention.

Fragment

A fragment is suitably at least 10 amino acids in length, suitably at least 25 amino acids, suitably at least 50 amino acids, suitably at least 100 amino acids, suitably at least 200 amino acids, suitably the majority of the polypeptide of interest such as the photosensor polypeptide of the invention. Suitably a fragment comprises a whole motif or a whole domain of the photosensor polypeptide of interest.

Sequence Homology/Identity

Although sequence homology can also be considered in terms of functional similarity (i.e., amino acid residues having similar chemical properties/functions), in the context of the present document it is preferred to express homology in terms of sequence identity. Sequence comparisons can be conducted by eye or, more usually, with the aid of readily available sequence comparison programs. These publicly and commercially available computer programs can calculate percent homology (such as percent identity) between two or more sequences.

Percent identity may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are
performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in percent homology (percent identity) when a global alignment (an alignment across the whole sequence) is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology (identity) score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology/identity.

These more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with a few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum percent homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA (Altschul et al., 1990, J. Mol. Biol. 215:403-410) and the GENEWORKS suite of comparison tools.

Although the final percent homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied. It is preferred to use the public default values for the GCG package, or in the case of other software, the
default matrix, such as BLOSUM62. Once the software has produced an optimal alignment, it is possible to calculate percent homology, preferably percent sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

In the context of the present document, a homologous amino acid sequence is taken to include an amino acid sequence which is at least 50, 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level. Suitably said comparison is made over at least 50 or 100, preferably 200 or 300 amino acids with any one of the relevant polypeptide sequences disclosed herein, most suitably across the full length of the polypeptide of interest. Suitably, homology should be considered with respect to one or more of those regions of the sequence known to be essential for protein function rather than non-essential neighbouring sequences. This is especially important when considering homologous sequences from distantly related organisms.

The same considerations apply to nucleic acid nucleotide sequences.

The term 'derived from' has its normal meaning in the art, wherein a substance is considered to be 'derived from' a first substance when part of the substance has been created or constructed through a chain of events which incorporates all or part of the first substance into the substance in question. Naturally the two substances are likely to differ eg. through mutation, addition or deletion or similar modification, but if the substance in question has inherited features from the first substance then it is derived from it. In particular, when used in connection with biopolymers such as polynucleotide(s) or polypeptide(s), a substance is considered to be derived from a first substance when it possesses sufficient sequence identity to be recognised as related to the first substance. In this context, if a substance is derived from a first substance, then said substance preferably has at least 10 contiguous residues which possess at least 40% identity, preferably 50% identity, preferably 60% identity, preferably 70% identity, preferably 80% identity, preferably 90% identity, preferably 95% identity, preferably 96% identity, preferably 97% identity, preferably 98% identity, preferably 99% identity or even more. Preferably said substance has at least 15 contiguous residues with said identity, preferably at least 20 residues, preferably at least 30 residues, preferably at least 50 residues, preferably at least 100 residues, preferably at least 200 residues, or even more. For multimeric entities, the term may be applied to the complex and/or to individual components as will be apparent from the context. Generally it will be enough if one of the subunits is derived from the given entity.
Cell Production

Suitably the cells of the invention are produced in vitro.

It is an advantage of the invention that the cells can be produced from any cell line such as an ES cell line. The starting cells such as ES cells are transformed/transfected with selectable markers as described and then the desired cells can be easily purified by cell sorting on the basis of those markers after differentiation. This is explained in more detail below.

Suitably the cells of the invention are derived from stem cells by inducing differentiation of the stem cells and then selection of the cells of interest from the pool of induced stem cells. Thus typically cell production comprises a first step of generating the cells (e.g. by induction of differentiation in a pool of starting cells such as stem cells) and a second step of selection (sorting/purifying) those cells of the desired type from amongst the pool of induced/differentiated cells produced from the starting stem cells.

For example, the motor neurons of the invention may be produced by inducing differentiation in a pool of embryonic stem cells (ESCs) or of pluripotent stem cells such as induced pluripotent stem cells (iPS/iPSCs), and then selecting for those which have assumed a motor neuron fate i.e. those which express gene(s) under the control of a motor neuron specific promoter.

This applies equally to production of other cell types such as astrocytes, oligodendrocytes or others. Clearly the differentiation protocol and selection protocols will involve different reagents appropriate to the particular cell types being produced/selected, but in principle the overall method is the same for each type of cell being produced.

Pluripotent embryonic stem cells (ESCs), which were first described in mice about 25 years ago, have since revolutionized mammalian genetics. ESCs are derived from the 'inner cell mass' of blastocyst-stage embryos and can be cultured and genetically manipulated in vitro. When reintroduced into early embryos, ESCs contribute to all tissues of the resulting organism, including the germline. Traditionally, ESC technology has been mostly used to generate mice carrying loss-of-function mutations. In this approach, the ESCs are transfected with a gene targeting construct carrying sequences homologous to the gene of interest and a selection marker. During homologous recombination in ESCs, parts of the target gene are replaced by exogenous DNA, which results in a null allele of that gene. The genetically altered ESCs
are then used to generate chimeric mice. Following germline transmission, the null allele is bred to homozygosity, and the phenotype of the mutation can be studied in vivo. The gene targeting technology has been refined to allow the conditional inactivation of genes in specific cell types and/or at specific time points using the Cre/loxP recombination system.

The recent development of human ESC lines and Induced pluripotent stem cell (iPSCs) promises to transform ESCs from a basic research tool into a medical technology that, in principle, could be used to replace any tissue which is lost due to injury or degenerative disease in humans. iPSCs, in particular, hold tremendous potential; these ESC-like stem cells are generated from differentiated somatic cells though the transfection of four transcription factors. Because iPSC can be produced from a patient's own cells, they are tolerated by the recipient's immune system, in contrast to conventional transplants.

In vitro differentiation protocols for cell types that hold therapeutic potential for various diseases have been explored. These include hematopoietic stem cells (leukaemia), pancreatic beta-cells (autoimmune diabetes), dopaminergic neurons (Parkinson's disease) and motor neurons (amyotrophic lateral sclerosis). Remarkably, the extrinsic and intrinsic factors that direct ESCs into specific cell lineages often mimic normal developmental programs that drive differentiation during embryogenesis. For example, the in vitro derivation of motor neurons from ESCs, via a neural progenitor stage, is performed by the induction of this specific cell fate by the morphogens retinoic acid and sonic hedgehog, two factors that also drive motor neuron development during normal embryogenesis.

Suitably the cells are human cells. Suitably the human cells are produced from human stem cells (e.g. hESCs or human iPS). Suitably when the cells are intended for implantation into a human subject, the cells are produced from induction of cells such as iPS from a tissue matched subject, or more suitably from the same subject. This has the advantage of avoiding graft-vs-host or immunological problems with implantation. This has the further advantage of avoiding cross-infection or contamination from tissue samples e.g. virus infection or prion infection or other tissue-borne problems.

Suitably the starting cells, from which the cells of the invention are produced, are human cells.

For example a suitable cell for a starting cell is a human H9 cell (WiCell Research Institute, U.S. Mail P.O. Box 7365, Madison, Wisconsin, USA).
For example, a suitable cell for a starting cell is a human iPS cell from Pentao Liu at the Sanger Institute, Hinxton, Cambridge, CB10 ISA, U.K.

Suitably the starting cell is a patient's own cell.

Suitably each cell in the population is from the same species; suitably each cell in the population is human.

**Further Companion Cells**

Companion cells such as astrocytes are discussed herein. There may be advantages in providing further companion cells.

For example, long-term survival of derived motor neurons may be improved by additional accessory cells other than astrocytes. The most likely 'companion' cells are oligodendrocytes, which can also be generated from ES cells (see Brustle et al. 1999 Science vol 285 p754). Oligodendrocytes will myelinate the motor axons inside the device of the invention, as they would normally do for the central portion of motor axons in the spinal cord. There are surface antigens that can be used to sort (select/purify) oligodendrocytes or their progenitors from stem cells such as ES cell differentiation cultures. Exemplary antigens include those disclosed as in Cizkova D, Cizek M, Nagyova M, Slovinska I, Novotna I, Jergova S, Radonak J, Hlucilova J, Vanicky I. J "Enrichment of rat oligodendrocyte progenitor cells by magnetic cell sorting." Neurosci Methods. 2009 Oct 30;184(1):88-94. This document is expressly incorporated herein by reference specifically for the sections regarding enrichment and selection of oligodendrocytes.

It may be advantageous for the cell population to further comprise local spinal interneurons. The various interneuron subtypes are molecularly well-characterized and develop under the motor neuron-differentiation conditions. These can be sorted (selected/purified) as described for motor neurons, using markers appropriate to the interneuron subtype being selected.

**Induction/Differentiation**

The skilled worker will be aware that there are variants of the motor neuron differentiation procedure. The overall strategy is generally to insert a motor neuron-specific transgene into the ES cell genome. The pool of cells carrying the transgene is then induced to differentiate. The cells expressing the transgene are then selected. For motor neuron selection, suitably the transgene consists of a Hb9 promotor driving a selectable marker. This construct allows motor neuron detection and sorting.
The same approach applies to other cell types such as astrocytes or oligodendrocytes when produced in this manner, although of course the promoter will be varied accordingly. In summary, the marker transgene is introduced into the pool of cells; the pool of cells is then induced to undergo appropriate differentiation; the cells of interest (i.e. those expressing the selectable marker) are then selected from the pool of induced cells.

The promoter such as the Hb9 promoter can be introduced in any suitable form such as a short 5′ genomic fragment (e.g. as described in Wichterle et al. 2002 Cell Aug 9:10(3):385-97) or as a ‘bacterial artificial chromosome’ (BAC) construct. The BAC approach for human ES cells is suitably as described in (Placantonakis DG, Tomishima MJ, Lafaille F, Desbordes SC, Jia F, Socci ND, Viale A, Lee H, Harrison N, Tabar V, Studer L. Stem Cells. 2009 Mar;27(3):521-32 "BAC transgenesis in human embryonic stem cells as a novel tool to define the human neural lineage.").

A typical BAC, for example comprising a motor neuron specific promoter such as an MNX promoter, is RP24-35123 (‘mouse MNX1 BAC’).

As a selectable marker, in principle any gene capable of generating a detectable signal may be used. Especially suitable are surface markers, which have the advantage of being available for visualisation by antibody binding to the surface of the cell(s). Multiple markers may be used if desired. These may be under different promoters, under the same promoter, or as a fusion protein under a single promoter.

For example human CD2 may be used. Human CD2 may also be used fused to a fluorescent protein such as GFP. Alternatively human CD14 may be used, which has the advantage of providing a stronger signal.

If naturally occurring markers are used then advantageously the marker transgene may be omitted. In this embodiment, the starting cells are the same and the induction of differentiation is the same but the cells do not carry a marker transgene and the sorting (selection/purification) is carried out using the naturally occurring marker characteristic of the cell type which it is desired to produce.

When preparing cells for eventual use in humans, suitably non-human markers are omitted. Thus when the cells are for human use, suitably non-human markers are specifically excluded. GFP is an example of a non-human marker. Suitably markers are human markers.
We describe a differentiation protocol of astrocytes from stem cells such as mouse ES cells, adapted from a number of existing protocols in the literature. This is explained in detail in the examples. However it must be noted that in principle, most, and possibly all, neurogenic protocols produce some astrocytes. Therefore the induction procedure is not critical to the invention. Clearly the guidance given regarding sorting remains important so that cells of the desired type can be efficiently selected from the pool of cells undergoing neurogenic differentiation.

Astrocyte differentiation from mouse ES cells can be performed according to "Embryonic stem cell-derived glial precursors: a source of myelinating transplants." BrCistle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. Science. 1999 Jul 30;285(5428):754-6. This paper also describes the emergence of oligodendrocyte in the cultures. Therefore this document is incorporated herein by reference, in particular for the passages of this document which expressly detail the astrocyte differentiation procedure and the passages which address oligodendrocyte emergence.

Astrocytes may be sorted (selected) with a marker such as the GFAP::CD2-RFP marker transgene, similar to the strategy we outline for motor neurons. Alternatively, a MACS sorting kit based on an antibody directed against GLAST, a natural surface marker expressed on astrocytes mya be used, such as the one available from Miltenyi Biotec. This GLAST selection has the advantage that it can be used for astrocytes derived from any ES cell line.

Transfection
The light sensitive proteins can be introduced to the cells via transfection. This can be done for example with viral transfection technique using Adeno-associated virus (AAV), electroporation technique or other transfection techniques. The gene transfer of photo-sensor transgenes into undifferentiated ES cells or iPSCs can be performed in cell cultures. The transgene construct, consisting of an appropriate tissue-specific and/or inducible promoter, the photo-sensor transgene and suitably a selectable marker such as an antibiotic resistance cassette will be introduced into the cells by electroporation, Infection with recombinant virus or another suitable method. Stable transfectants will then be positively selected for antibiotic resistance (such as G418, hygromycin or puromycin), and either monoclonal or polyclonal ESC/iPSC lines will be established. The cell lines will be tested for expression of the photo-sensor transgene by in vitro differentiation from stem cells into specific neural subtypes. Positive stem cell lines will be expanded for the use in the invention such as in implants as described herein.
Cell Sorting

Any suitable means known in the art may be used to sort (i.e. select) the cells marked as described above. For example, cells may be sorted on the basis of a fluorescent marker (or a non-fluorescent marker which is subsequently stained with further reagent(s) to render it visualisable such as via fluorescence).

Alternatively cells may be selected or immobilised on the basis of their marker. For example, magnetic sorting may be used to separate/select cells on the basis of their marker expression.

For preferred markers such as CD2 and/or CD14, reagents for either fluorescent or magnetic sorting (e.g. Miltenyi MACS system) are commercially available.

It is advantageous to dissociate cells prior to sorting whilst retaining their CDs. This leads to efficient sorting using the surface antigens (CDs).

Advantageously the protease mixture used to dissociate the cells is selected to retain their CDs.

Photosensor

The photosensitive protein (sometimes referred to as 'photosensor') is one which is capable of triggering or suppressing an action potential upon illumination with light of cognate wavelength.

A single protein will typically possess only a single function with regard to the action potential i.e. the protein will either trigger or suppress an action potential upon illumination. Clearly it may be advantageous to use a photosensitive protein which can both trigger and suppress an action potential, for example upon illumination with lights of different wavelengths so that the function(s) can be discretely controlled. However, unless otherwise indicated, embodiments of the invention are discussed with single functions for single photosensor species.

Whether or not the photosensor triggers or suppresses an action potential depends on the nature of the photosensor.
For example photosensors comprising channelrhodopsin such as ChR2 and/or VChrl or melanopsin will depolarise the cell upon stimulation with cognate wavelength light and will therefore trigger an action potential. Without wishing to be bound by theory, this is because the stimulated protein acts as an open ion channel leading to depolarisation and stimulation of action potential.

For example photosensors comprising halorhodopsin (NpHR) will hyperpolarise the cell upon stimulation with cognate wavelength light and will therefore suppress an action potential. Without wishing to be bound by theory, this is because the stimulated protein acts as a chloride pump leading to suppression of depolarisation and suppression of action potential.

The skilled worker may easily choose photosensor proteins according to whether it is desired to stimulate or to suppress action potentials in application of the invention.

Suitably the photosensor polypeptide is provided by insertion of a nucleic acid encoding and capable of directing expression of same into the target cell.

The photosensor protein may in principle comprise light sensitive ion channels (e.g. channelrhodopsin + variants), light sensitive ion pumps (e.g. halorhodopsin) or light sensitive G protein cascades (e.g. melanopsin). More suitably the photosensor may be any photosensitive ion channel or gate protein, for example advanced versions of channelrhodopsin are being discovered from biology or engineered. Such engineering may red shift the required wavelength, as in volvox (Zhang F, Prigge M, Beyriere F, et al. "Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri". Nat. Neurosci. 11 (6): 631-3, April 2008) which is sensitive to green wavelengths.

Additionally, engineering can create bistable states, for example via modification of the cl28 amino acid (Andre Berndt, Ofer Yizhar, Lisa A Gunaydin, Peter Hegemann, Karl Deisseroth "Bi-stable neural state switches" Nature Neuroscience 12, 229 - 234 (2008), such modification can significantly reduce the light requirement. Similarly, for halorhodopsin, new variants such as that developed by Chow et al (Chow, BY; Han, X; Dobry, AS, et al. "High-performance genetically targetable optical neural silencing by light-driven proton pumps" NATURE Vol 463(7277), pp98-102, 20(0) similarly show advances in performance. It may also be that the excitatory and inhibitory proteins are fused into a single bi-active protein (Han X, Qian X, Stern P, Chuong A and Boyden ES (2009) Informational Lesions: Optical Perturbation of Spike Timing and Neural Synchrony Via Microbial Opsin Gene Fusions, Frontiers in Molecular Neuroscience, 2:12).

In a preferred embodiment the photosensitive protein comprises a light activated G-protein linked cascade. An example of this is melanopsin, suitably human melanopsin
(Melyan Z, Tarttelin EE, Bellingham J, Lucas RJ, Hankins MW "Addition of human melanopsin renders mammalian cells photoresponsive" Nature 433 (7027): 741-745 Feb 17 2005) which exists naturally in some of the retinal ganglion cells of the eye. As such, G-protein linked systems have inherent amplification, they considerably reduce the required light intensity. An advantage of such a system is that as melanopsin already exists in the human genome, it does not result the genetic modification of humans to express foreign genes. Engineering of melanopsin may improve upon the speed of photoresponse.

Thus for some applications melanopsin may be more suitably used because it already exists in the human genome and thus human rejection issues would not be lessened or eliminated. Use of melanopsin would have further benefit(s) at ethical approval stages.

In one embodiment the photosensor may be a chemical modification of existing ion channels or receptors. An example of this is by Volgraf et al (M. Volgraf, P. Gorostiza, R. Numano, et al. "Allosteric control of an ionotropic glutamate receptor with an optical switch" Nature Chemical Biology Vol 2(1) pp47 - 52 Jan 2006), who modified a glutamate receptor to be light sensitive. Such systems do not require genetic modification of the cells, but may require continuous perfusion of new photosensitization agents as existing ones decay.

By 'cognate wavelength' is meant the wavelength of light to which the photosensitive protein responds. Individual photosensitive proteins have corresponding individual light sensitivities. For example, photosensitive proteins comprising halorhodopsin (NpHR) typically react well to yellow light (e.g. wavelength approx 580nm); photosensitive proteins comprising channelrhodopsin (ChR) typically react well to blue light (e.g. wavelength approx 470nm). Thus the term 'cognate' is to be understood with regard to the photosensitive protein being targeted. In this sense cognate means a wavelength of light effective in stimulation of the photosensitive protein.

Thus, the triggering/delaying of the neural activity, and subsequent muscle contractions, with light can be achieved via light sensitive ion channels and light sensitive ion pumps. Presently the channelrhodopsin family, which are light gated ion channels provide the best method of optical triggering, whereas the halorhodopsin family of light gated ion pumps provide the best method of delay. When depolarizing light (light that activates the ion channel) is turned on, it triggers the opening of the light sensitive ion channels and the influx of positive ions (cations) into the cytoplasm. The result is depolarization of the neurons that, when it overcomes a specific threshold,
activates the natural spiking and contraction mechanism of the cells. Typically this mechanism involves a strong influx of Na⁺ into the cell from the activation of the voltage-gated calcium channels.

In the opposing inhibitory scenario, when hyperpolarizing light flight that activates the sensitive ion pumps) is turn on it triggers the pumping of the light sensitive ion pumps and an influx of negative ions (anions, such as chloride) that hyperpolarize the cells. This inhibits or delays depolarisation of the cells. By artificially controlling the depolarizing and hyperpolarizing of the neurons a precise signalling rhythm can be precisely regulated.

As an example, embryonic stem cells differentiated into neurons by expressing a light sensitive ion channel ChR2 and a light sensitive ion pump NpHR that can be independently activated due to a difference in their wavelength sensitivity. A pulse of blue light (~470nm) will trigger the opening of ChR2 and an influx of monovalent (such as sodium) and bivalent (such as calcium) cations that depolarizes the cell. A pulse of yellow light (~580nm) will selectively activate the NpHR and an influx of anions, hyperpolarizing the cell.

In some embodiments ChR2 is preferred. ChR2 offers a fast response time. ChR2 works well as demonstrated herein.

Photosensor protein variants
Photosensor proteins mentioned herein may be enhanced or modified as is known to a person skilled in the art, e.g. via mutation.

For example, photosensor polypeptide variants may enhance the proteins from their parental or wild-type form according to the following guidance:

1. Channel conductivity - Channelrhodopsin has a conductivity of 50fs whereas a glutamate channel has a conductivity of ~1ps. Thus there is scope for improvement.

The more conductivity, the greater the impact of the opening of an individual channel. This has the advantage of reducing the light requirement.

2. Kinetics - variants such as C128 can make bistable channels allowing channels to remain open until an off pulse turns them off. This is particularly significant in systems with slow dynamics such as described herein, as it has the advantage of reducing the light requirement. However care must be taken in use of the C128 mutation (variant) because this can be slow to turn off. Therefore in some embodiments suitably the C128 mutation is specifically omitted; suitably the wild type residue is used at the position corresponding to C128.
3. Colour - variance of absorption spectrum can be used to make protein variants which respond to different stimulatory or inhibitory wavelengths.

4. Melanopsin - modification of this protein and/or its cascade components could improve speed, presently up to 10s response times. The use of melanopsin can reduce the light requirement compared to ChR2.

Applying the same principles, modification of the genetic expression could include:

1. Improved promoters - i.e. better expression
2. Fusion expression (e.g. ChR2 and NpHR) which has the advantage of allowing simultaneous expression of both excitatory and inhibitory groups.

In more detail, specific improvements which may be targeted include:

1. Chromatic range: Having a choice of different colours may allow us to target different circuits with different colours of light. E.g. we could use blue light to target an excitatory circuit and red light (NpHR) to inhibit. We could use green light to target an inhibitory circuit and orange light to inhibit the inhibitory circuit.

2. Light requirement - every action to improve the impact of each photon via channel conductivity or otherwise will reduce the quantity of light required to achieve the desired neural signalling. That will reduce the light required from the emitter, which often will have a nonlinear efficiency vs illumination. This in turn will reduce the power requirement from the battery or wireless power source which can also be nonlinear. As such small reductions in destination energy can have large repercussions on the battery/power. This may be important from an aesthetics and surgical perspective.

3. Speed - for our preferred applications the information stream is effectively in the Hz frequency range - i.e. regular pacemaker function, whether the heart or breathing tends to be around once per second. Thus our kinetics can be slower than the higher speed ~50Hz tonic signalling of sensory neurons (in the eyes, ears etc). However it is still advantageous to have reasonably fast response times (better than 100ms) in order to turn things on and off sufficiently. As such if Melanopsin could be improved to this time period, or used in some form of sinusoidal pattern around a functional threshold, then this may be advantageous.

It must be borne in mind that these factors may affect each other in terms of function (e.g. channel conductivity of 1fs AND standard expression of ~100million molecules per cell creates a light requirement of ~1uW/mm2). Thus the most suitable photosensor or photosensor variant should be chosen according to the application of the invention.
Clearly not all variants which might be made are necessarily improvements. Some variants, whilst being less functional in absolute terms compared to existing or wild type sequences may nevertheless be usable in the invention. If there is any doubt as to the applicability of a particular variant then it may be tested, for example as set out herein, to determine its suitability. In particular, variants acquiring or accentuating the following properties are advantageously avoided: Low expression; Low light absorption; Slow kinetics; Poor channel conductivity; Poor channel specificity (i.e. ChR2 preferentially allows sodium ions to come through, even though it is termed non-specific in the literature. If there is a lack of specificity, then there will be a large impact on function.) Poor expression; Expression in places other than the cell membrane; Immune response (N.B. so far no one has reported a significant immune response to ChR2 or NpHR but care must be taken when expressing in humans - an inflammatory response would be undesirably.)

Photosensor Transgene

In addition to the discussion of this above, the photosensor transgene is now described in more detail.

By way of example, preferred photosensors have the amino acid sequence encoded by the following preferred constructs for ChR, NpHR and melanopsin expression respectively:

ChR2-H134R sequence:

```
ATGACATGCGCCTTTCTGCCTCGCCGCACCGAATCTTTTGTGCTGTACTACCCCTTGTGGTGGGGTACGACGTGGTTACCCAAAGGGAGTTGTTCGAGATATCCGCAAAACCACCAAACTGAACATCGGCGGAACGGAGATCGAGGTCGAGACTCTCGTCGAAGACGAAGCTTTGTCCIGTCATCCTTATCCGCCTGAGCAACCTCACCGGCCTGAGCAACGACTACAGCAGGAGAACCATGGGACTCTAGGACTATGGCGGCGCTTTGTCTGCCGTCGGACGCGAACTTTTGTTCGTTACTAATCCTGTGGTGGTGAACGGGTGGGATGTTCCATCTGCTTCTGGGACCCCGGCTGATGATTAA
```

NpHR sequence:

```
ATGACAGAACATCCTCCCTGTCCCCTGACCGAGAGGTTACCCCAAAAGGTTAAGGGATATTGCCGCTTTCTGCCTCTTGCCGTCGCTGGCCGCCACCGCCGGAGACAGCCTTTTGTGCTGTACTACCCCTTGTGGTGGGGTACGACGTGGTTACCCAAAGGGAGTTGTTCGAGATATCCGCAAAACCACCAAACTGAACATCGGCGGAACGGAGATCGAGGTCGAGACTCTCGTCGAAGACGAAGCTTTGTCCIGTCATCCTTATCCGCCTGAGCAACCTCACCGGCCTGAGCAACGACTACAGCAGGAGAACCATGGGACTCTAGGACTATGGCGGCGCTTTGTCTGCCGTCGGACGCGAACTTTTGTTCGTTACTAATCCTGTGGTGGTGAACGGGTGGGATGTTCCATCTGCTTCTGGGACCCCGGCTGATGATTAA
```

OPN4 sequence:

```
ATGACAGAACATCCTCCCTGTCCCCTGACCGAGAGGTTACCCCAAAAGGTTAAGGGATATTGCCGCTTTCTGCCTCTTGCCGTCGCTGGCCGCCACCGCCGGAGACAGCCTTTTGTGCTGTACTACCCCTTGTGGTGGGGTACGACGTGGTTACCCAAAGGGAGTTGTTCGAGATATCCGCAAAACCACCAAACTGAACATCGGCGGAACGGAGATCGAGGTCGAGACTCTCGTCGAAGACGAAGCTTTGTCCIGTCATCCTTATCCGCCTGAGCAACCTCACCGGCCTGAGCAACGACTACAGCAGGAGAACCATGGGACTCTAGGACTATGGCGGCGCTTTGTCTGCCGTCGGACGCGAACTTTTGTTCGTTACTAATCCTGTGGTGGTGAACGGGTGGGATGTTCCATCTGCTTCTGGGACCCCGGCTGATGATTAA
```

29
Where possible, human photosensor proteins should be used. These have the advantage of avoiding or reducing immune reactions to a non-self protein. Thus the human melanopsin gene (OPN4) is preferred for this reason. In the examples, mouse Opn4 may be used: the properties of human OPN4 are expected to be very similar.

Clearly for photosensors derived from algae or bacteria (ChR2, NpHR), it is not possible to use the corresponding human polypeptide, as there are no homologs of these proteins in the human genome.

Suitably constructs for use in humans are codon optimised for humans. Clearly no codon-optimisation may be needed for genes which are already human.

In human patients, it is desired to avoid non-human genes/proteins as much as possible. Therefore, when ChR2 is used as the photosensor, suitably this is used without the YFP portion. For the same reason, suitably other fusion partners that facilitate detection of the photo-sensor transgene are avoided, such as HA- or Myc-epitope tags. See also: ChR2 sequence ChR2-H134R herein.

In addition to the promoter and the CDS of the photosensor, the transgene construct should contain:

1) 5' splice substrate upstream of the CDS

Exemplary Sequence 5'splice substrate:

```
GGCCGCGccTGCGCTGCGGAGGcAGCT^GGGGTGAGTACTCccTCTCAAAAGCGGGCATGAcncTG
```


In addition to the promoter and the CDS of the photosensor, the transgene construct should contain:

1) 5' splice substrate upstream of the CDS

Exemplary Sequence 5'splice substrate:

```
GGCCGCGccTGCGCTGCGGAGGcAGCT^GGGGTGAGTACTCccTCTCAAAAGCGGGCATGAcncTG
```

2) **poly-adenylation signal** (such as bovine growth hormone pA) following the CDS.

Exemplary Sequence bGHpA:

```
TAGTGGCATGGGGCGGCGCTAGCTAGCTGATCAAGGGGCGACTGCGTTCTTCTAGTGGCAGCCATCTGT
TGTTGCCCGCTCCGCCGCGCTCTTTGAGACCCTGAGGAAGGTGGCAGCTCTGCTTTTCTAGAAGGAGGAGG
AGA
```

3) **Selectable marker** driven by a promoter that is active in ES cells. The marker would be typically placed downstream of the pA signal. The selection cassette should ideally be surrounded by recombination sites that allow its excision from the ES cell genome. An exemplary selection cassette could be a neomycin resistance gene with a ubiquitous promoter (such as PGK, TK or CMV) flanked by FRT recombination sites. This cassette can be deleted by transient expression of Flp recombinase to avoid interference with photosensor-expression.

Here is an example sequence for such a cassette:

```
Sequence FRT-neo-FRT:
```

```
TTATTACCCGCGGGAATGTTCTTATCGCAGTTTCTTTTTTTAATATTACCTAAATATAGGAACCTTCCGTAGGGGAC
TTTGCGCGGAATTGCGGAGGACCCCTATTGTGTTAATTTTTTCTAAATATTCTAAATATAGGAACCTTCCGTAGGGGAC
AATACGCTGAAATGGTGTTAATATTCTAAATATAGGAACCTTCCGTAGGGGAC
```

In addition to the three elements outlined above, the transgene construct may optionally also contain Transposase recognition sites (optional). The Tol2 transposase may suitably be used to stably integrate BAC constructs into ES cells. The idea is to co-transfect a BAC construct that carries tol2 target sites in the vector backbone and the Tol2 transposase, which will then mediate insertion into the ES cell genome. It may be advisable to subsequently map the integration site to ensure that no known tumour suppressor gene has been inactivated by the transposition event. If this method works, it would allow the integration of single-copy, complete BACs into sites of active gene expression. This would help increase the likelihood that the transgene (surface marker and/or photoreceptor) is expressed and shows the expected expression pattern. Suitably the tol2 transposase and related elements are as available from the National
Institute for Genetics, Japan (Reference Tol2: Suster ML, Sumiyama K, Kawakami K.
Transposon-mediated BAC transgenesis in zebrafish and mice. BMC Genomics. 10:477).
Alternatively other readily available transposases such as piggyBac or SleepingBeauly could be used as an alternative.

**Transgene Promoter**
The system may be used with a doxycycline-inducible promoter, however this promoter is i) often silenced in postmitotic neurons and ii) would require constant, long-term activation by doxycycline in the animal model / human patient. Therefore other promoters may be more suitably used. For example the photosensor may be placed under the control of the tau (Mapt) promoter, which is active (and stably expressed) in postmitotic neurons, but not ES cells. This may be done (e.g. in mouse ES cells) by inserting the tau::ChR2 construct by gene targeting in ES cells, though BAC transgene constructs could also be used. Both approaches are, in principle, also applicable to human ES cells. In general, the strategy of using neuron-specific rather than inducible promoters is advantageous, as expression is excluded from ES cells but then locked in once they have been differentiated into motor neurons (or other neurons).

**Introduction of Nucleic Acids**
With the appropriate method (e.g. electroporation), very large constructs, such as recombinant BACs, can be transferred and integrated into ES cells. The BAC constructs which are suitably used for the celltype-specific sorting marker (Mnx1 - motor neurons; GFAP - astrocytes) and/or for the photosensor transgene (Mapl) are typically 100-200KB in size. Lipofection has been described as an alternative method to transfer BACs into cells.

**Preparation of Cell Population**
The cell population of the invention is suitably made by combining the different cell types in the desired ratio.

It may be advantageous to aggregate the cell population. This may be done for example by combining the cells in a 'hanging drop' method. This may be carried out for example as in "The outgrowth of the nerve fiber as a mode of protoplasmic movement." Harrison RG. 1910. J Exp Zool 9:787-846, or as in any of the many subsequent papers citing this publication, such as "Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems." Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Biotechnol Bioeng. 2002 May 20:78(4):442-53.
The ratio of motor neurons to astrocytes suitably corresponds to the ratio in normal human brain (1:10). This may be experimentally optimised. For other species a corresponding ratio should be chosen (non-human species typically have a lower ratio).

The volume per drop in the 'hanging drop' method is typically 10-20 µl.

**Implantable Device**

In one aspect, the invention relates to an implantable device comprising a population of cells as described above, wherein said device is structured to retain the cell bodies within the device.

Of course the device may be manufactured and sold independent of introduction of the cells. For example the device may be manufactured and sold before the cells are introduced. Therefore in another aspect, the invention relates to an implantable device capable of receiving a population of cells as described above, wherein said device is structured to retain the cell bodies within the device.

Suitably the device comprises optically conductive material. Cells and/or tissue typically do not conduct light very well, especially of the bluer wavelengths which may be used in the invention. Thus it is an advantage that the light be transmitted through the structure of the device. It is further advantage of transmission of light through the structure of the device that the light can be more evenly distributed in intensity to the target cells.

Suitably said optically conductive material has a high refractive index. By 'high refractive index' is meant sufficient to promote total internal reflection of light. This is advantageous in transmitting the light to the cell bodies within the device in use. This minimises losses to the surrounding tissue. The refractive index of the surrounding tissue or fluid is similar to that of typical optical fibres, which is 1.5. Most suitably the refractive index of the conductive material is 1.8 or more.

In an alternative approach multilayer cladding could be used to separate the light conductive core from the surrounding tissue. Suitable outlet points can then be created to allow the light to access the cells.

Suitably said device is formed as a light guide to conduct light from an input to the retained cell bodies. The light input may be a source forming part of the device, for example a light emitting diode or other similar light generator. Alternatively the light input may be in the form of
an optical fibre or other source coupled to the device of the invention which may permit more flexibility in provision of light.

The device is advantageously constructed as a cage in order to retain the cell bodies. By cage is meant a branched or mesh wall structure. This has the further advantage of acting as a branched light guide. This has the further advantage of more evenly distributing the light. Motor neuron cells are typically 10-20 μm in diameter. Axons from said cells are typically approx 1 μm in diameter. Therefore the cage structure is suitably chosen to retain the cell bodies but provide pores to permit extension of axons out of the device.

A further advantage of the cage structure is to retain each of the cells in the population. This has the medical advantage of preventing any of the cells in the population escaping into the subject in which the device is implanted. This is advantageous in the situation where a cell of a different type may have accidentally been included in the population e.g. an undifferentiated embryonic stem cell or other undesirable cell type and is therefore retained in the device by the cage structure rather than entering the patient's tissues.

A further advantage of the cage is that the implanted cell bodies need to be attached or anchored, otherwise they may migrate (e.g. migrate along their own axons). This is important from a safety perspective and also from a practical perspective in that the cells need to be localised in order to be stimulated by light directed to their location.

The device, such as the cage part of the device, may be made from any optically conductive (such as optically transparent) material e.g. poly di methyl sulphonate (PDMS) or polyimide.

The device may comprise an optical fibre as light source.

The device may be single compartment or multi compartment. An advantage of a single compartment construction is if may promote synchrony. A further advantage of a single compartment is that the neurons are more likely to be linked by synapses which can promote strong signalling. Furthermore, it is an advantage if the neurons are linked by synapses because the effects of any cells failing to express the photosensitive protein transgene are advantageously ameliorated since any non-expressers or poor expressers are likely to be 'fired' by their neighbours via the synaptic links following stimulation by light to the device.
Suitably the device comprises pores to allow extension of axons outside the device. Pores should be so sized as to retain the cell body but permit axon extension. Thus pores are suitably 1-5\(\mu\)m in diameter, suitably 1-3\(\mu\)m in diameter, more suitably 1-2\(\mu\)m in diameter, most suitably 1.5\(\mu\)m in diameter. The shape of the pore may be any suitable shape; the fact that the dimensions are discussed in terms of diameter should not imply a perfect circular pore shape. Suitably the pores are any shape convenient to manufacture which permits axon extension; in non-circular pore shape the diameter may be taken as the maximum dimension of the pore opening.

Separate from any pores, the device may comprise perforations. Perforations are apertures or gaps in the material of the device which permit molecular exchange with the medium surrounding the device. This is important for example to permit feed/oxygen molecules in and waste/carbon dioxide molecules out. A perforation is too small in size to allow axon extension. Typically a perforation is 0.5\(\mu\)m or less in diameter. Perforations may be any shape such as slits, holes or other structures. The 'diameter' should be understood to be the maximum dimension of the perforation aperture. Perforations may not be necessary if molecular exchange is sufficient via the pores.

Alternatively the device may comprise permeable material. This has the advantage of permitting molecular exchange with the medium surrounding the device but at the same time does not permit axon extension. Devices comprising permeable material may not require perforations and vice versa; devices may comprise both permeable material and perforations.

Suitably the device further comprises an anchor. The anchor may take any suitable form known in the art, for example one or more hooks. The anchor may be made of any suitable material such as titanium, titanium oxide, hardened plastic or other material, provided always that the material is biocompatible.

The device may further comprise a biocompatible bioadhesive.

The size of the device is typically 100 \(\mu\)m to 200 \(\mu\)m wide.

The device may be any suitable shape. Most suitable is a a closed hollow tube with pores in one end, such as a bell shape with pores in the flared end. These have the
advantage of promoting directional axon extension. These have the further advantage of making it easier to implant and/or to couple to a nerve in the subject.

The device may further comprise an electrode for data collection. Such electrode(s) may be fabricated from traditional bioelectrode materials (such as Pt, Ir, or Ir02), or more modern bioelectrode materials such as carbon nanotubes or organic synthetic metals. The latter may provide advantages in electrode performance and lifetime.

The cell population is suitably introduced into the device from the 'hanging drop' stage of cell population production. This may suitably be direct introduction of the hanging drop into the compartment of the device for receiving the cells. Alternatively, the cell population may be injected as a cell suspension into the device, for example with a capillary. Closure of the of the device in order to retain the cell bodies (i.e. to close or complete the cage) may be achieved via skilled or robotic manufacture, e.g. whereby a lid is connected in place with adhesive and/or physical clasp. Alternatively a MEMS self assembly approach may be used to achieve self fabrication of the final cage structure upon cell placement.

To facilitate production, the cells may be placed into the body of the device through a mouth, which mouth may then be sealed with a membrane such as a permeable membrane. The mouth may be of any suitable size to permit easy introduction of the cells. The mouth is sealed in use so is not itself considered a pore or perforation, although of course the wall or membrane used to seal the mouth may comprise pore(s) or perforation(s) as required.

The number of motor neurons per device will be determined by the particular application. When the device is coupled to a nerve in vivo, ideally the number of motor neurons in the device is approximately half the number of motor neurons in the nerve. For example when the device is coupled to the phrenic nerve, it will typically contain a few hundred motor neurons such as 300 motor neurons when the phrenic nerve typically contains several hundred motor neurons such as 600 motor neurons.

Suitably the device contains at least 100 motor neurons.

The light source/light input for the device of the invention is suitably provided and/or controlled by any suitable means known in the art. Controllers and light sources are well known and thus a skilled worker may easily incorporate or couple such items to the device of the invention.
The electrical activity of the implanted neurons and/or related neurons can be sensed and fed to the control unit of the device. Further sensing capabilities can be used to modulate the light stimulus of the device, this include mechanical sensors such as accelerometers, further ECG analysis including QT-interval, minute ventilation sensors, and biochemical sensor for analytes such as glucose, lactate, pH, free fatty acids, amino acids, carbon dioxide and oxygen. There is typically no interference between light stimulation and the electrical recording from the electrodes, so they can be performed simultaneously on any desired area. Nevertheless, the electronics comprising for example the amplifier circuitry may be shielded from the light to ensure that interference is reduced or eliminated. An alternative way of monitoring the electrical activity of the neurons in the implant is to equip them with a genetically encoded calcium sensor. For example, when the stimulatory light is blue, by choosing such a sensor to work in the red spectrum, the signal may be read out through the optical fibre cables and would not interfere with the (e.g. blue) light stimulus.

The device may have two further modules: a control unit and a multiple interfacing unit. The control unit may consist of a power supply, data acquisition and processing, an oscillatory controller for the light emitters, light sources, optics to extract the light from the source, optical sensors, and a communications interface to the outside world (e.g. RF or IR link). This unit is sealed in a biocompatible and electromagnetically isolated capsule and can be placed distant from the implant. It will also be small enough to be implanted subcutaneously so as not to interfere with the user’s daily life. The interfacing unit may consist of optics, light sources and local power supply/storage (optional), sensing facilities and potentially local processing and positioning and fixation facilities.

Light source & optics
Due to the low efficiency of the light sensitive proteins, i.e. low change in membrane potential vs. light flux, high photon flux is required for the photostimulation process at present. This can be realized for example with miniature high power light sources such as light emitting diodes (LED) or lasers. Organic light emitting diodes (OLED) are highly efficient and thus would provide and ideal candidate if they can achieve good long-term durability and sufficient brightness. At present however Gallium Nitride based light emitting diode provide the best solution. Each stimulation spot can be accessed with multiple light sources to address the difference in the spectral sensitivity of the proteins and to enable safe redundancy in illumination. Typically optical power in the range of 1pW/cm² to 100nW/cm² is required depending on the efficacy of genetic
manipulation, the efficiency of the photosensitization agent, and the efficiency of the optical delivery system. This requirement may be reduced with alternate melanopsin(s) to below 1pW/cm².

The light sources can be placed in the interfacing unit or in the distant control unit. In the second case the light is coupled to light guides such as optic fibres and directed to the interfacing unit where it is focused/imaged on the required area. Microlenses and photonic crystals can be used to increase the light extraction yield in both the coupling and illuminating ends.

As an example, an array of high-power semiconductor based blue 470nm and 570nm micro-LEDs can be used to stimulate ChR2 and NpHR (2008 Poher V, Grossman N, Kennedy GT, et al, Micro-LED arrays: a tool for two-dimensional neuron stimulation, J PHYS D APPL PHYS, 2008, Vol:41). The two wavelengths can be realized on a single chip with sophisticated growth procedure, by the use of a fluorescent shifting technique, or can be realized on separate chips and combined optically.

The light is couched from the control unit casing to and guided to the interfacing units using optic fibres for example. A depolarizing light source (blue in case of ChR2) and a hyperpolarizing light source (yellow in case of NpHR) -if required, are dedicated to a single interfacing point (a redundancy of LEDs per interface unit will be realized for safety reason). The two light sources are coupled to a single optic fibre using microlenses, optic fibre fusion or photonic crystals waveguides. Alternatively, separate optic fibres can be used and their illumination can be optically overlaid in the interfacing unit.

The system can be cooled with heat sinks and Peltier cooling in order to reduce the temperature and increase the efficiency of the light sources.

In the interfacing units the light from the optic fibres is directed to the transfected cells using for example a micro-lens structure on the fibres tips. The optic fibres can inserted through the pericardial sac or any interfering tissue to increase the illumination efficiency.

**Sensing**

The downstream stimulated activity can be monitored primarily by integrating transparent electrodes such as Indium Tin Oxide (ITO), Poly(3,4-ethylenedioxythiophene) or carbon nanotube coated ITO. The electrical information can be used to optimise the optical stimulation requirement on the photosensitized neuron-differentiated stem cells.

Being an electro-optic device, it is feasible that an optical blood gas sensor can be incorporated, sensing the difference in the absorption of oxygenated and free haemoglobins at near-infrared light (600nm-800nm). This would provide a measure of
efficacy for both cardiac and phrenic nerve prosthesis, which could assist in the regulation appropriate breathing and heart beat.

In addition to such spectroscopic methods there may be potential for localised release of optically active materials that change their optical characteristics upon interaction with a target. It would thus be possible to sense the presence of the target optically. Such optically active materials could exhibit luminescent, fluorescent or absorption based changes. The interaction could be immunological or more low level, e.g. with aptamers or amino acid sequences. Additionally carbon fibre electrodes offer the potential for long term sensing without fouling. In this situation, the carbon fibre electrode would be used in cyclic voltammetric mode to determine the concentrations of targets via oxidation and reduction potentials.

Power supply
Due to the relatively slow temporal resolution of the lung (in comparison to the optoelectronic switching time) and due to the required delays between the different interfacing locations, it is likely that only a single light source will be required at a given moment, relieving the peak power requirements. Thus, our system can be powered via a small battery. Additionally, as the control component of our proposed device can be implanted subcutaneously, a radiofrequency or infra-red recharging system could be used to maintain battery levels.

In addition, there may be potential for energy scavenging to help in the return power to the energy storage unit (eg battery or charge capacitor). Such energy scavenging could include mechanical energy scavenging via MEMS systems optimized to the frequency of the heart and/or lung motion. Another embodiment may include chemical scavenging to extract energy from glucose, ATP or other sources in the blood stream. In the case of the mechanical system, the lung could provide an ideal location. In our embodiment we pulse individual micro-LED's between 0.5-1.0mA at 5-7V for 1-10ms. The total energy consumption of the light emitter in this case is 2.5microJ - 700microJ, resulting in a continuous power consumption of 2.5 - 700 microW when pulsed at 60 pulses/minute. The variation is dependent on the efficiency of the emitter, the optics and the level of sensitization of the cells. This is smaller than the present requirement for current electrical based pacemakers (Mallela, 2004).

In the case of ICD devices, present electrical ICD consumes several joules of energy, while in the case of the optogentic ICD device several millijoules will be enough. Thus our proposed device has considerable advantages with regards battery power consumption. Thus it will subsequently be possible to reduce the size of the required device.

39
The power requirement can be easily realized with conventional batteries such as Lithium Iodine or lithium carbon monofluoride (CFx) that have a very low self-discharge rate resulting in a long shelf life and a stable voltage throughout their lifespan, resulting in safe prediction of working time.

5 **Control Electronics**

The device may comprise electronics to interpret the sensing modules and control the optical stimulation. The system can be built from discrete components or from a single monolithic chip. Additionally individual processing components can be implemented either in analog or digital processing modes. The advantages of the former are extremely low power consumption and the advantages of the latter are accuracy and tunability. Examples of digital processing platforms include programmable logic such as CPLD (complex programmable logic device), PIC (Programmable interface controllers) or FPGA (Field Programmable Gated Arrays) and ASIC (Application Specific Integrated Circuits) platforms such as CMOS Complementary Metal Oxide Semiconductor.

Some of the system components may remain dormant for much of the time in order to save power. Thus it is preferable to maintain power to the sensor and preprocessing components. These can be operated using low power analog circuit methods. Alternatively they can be formed from analog to digital conversion and digital processing elements. A threshold can be implemented to only ensure the transmission of information to the main processing block when a safety limit is indicated. Alternatively this information can be transmitted directly.

The sensor processing component would have electrical isolation to ensure no electrolytic discharge into the solution. Where it is advantageous to monitor heart rate, the electric signal may require frequency analysis, and additionally, high, low and bandpass filtering to determine key characteristics of health. For other sensors, specific processing modalities include frequency, phase, amplitude, time domain, and differential analysis. Differential analysis is usually performed via a comparison between the main sensor and a control. Additionally for specific cases such as signal analysis from carbon fiber electrodes a signals would need to be analysed with variation in drive voltages. Furthermore, in the case of optical analysis, the signal may be analysed via the driving of a light based system and analysing the returning photons through a transducer such as a photodiode. Photodiodes in particular can be built into CMOS microelectronics. Furthermore there may in some cases be scope for inbuilt electrically controlled wavelength tuning systems which can be used to help perform spectroscopic sensing studies. In this case sensing would occur in conjunction with electronic scanning of the wavelength emitted or received.

The higher level processing module could compare the desired frequencies of
electrical activity in the stimulated motor neurons with that intended via optical stimulation. Where there is a mismatch in the electrical signalling, an intervention signal could be subsequently calculated. Additional information to such intervention may come via other sensors such as blood glucose. Where the invention is applied to phrenic nerve stimulation, such factors could feedback signalling to increase or decrease lung function. The intervention processing circuitry could be tunable via instructions stored in a memory module. This memory could be included in a dedicated ASIC but could also be part of a separate module with low power non-volatile memory. An appropriate stimulation frequency signal could then be sent to the light emitter controllers. The processing would in the first instance simply analyze whether the heart rate would fall into a specific frequency range and phase, but could also be expanded to consider all the features of the ECG signal in conjunction with other sensor information outlined in the sensor section. Additionally fuzzy logic and hysteresis could be implemented to ensure stability when the signals are on the borderline between intervention and non-intervention.

Given manufacturing variances between light emissive systems, the level of sensitivity of the photosensitized tissue, the electronics and how these change with time, a learning algorithm may be required. The learning algorithm circuitry would act to modify the stimulation signal in order to obtain maximum benefit. Thus, the learning circuits could in effect be part of a closed loop control. Alternatively the control could be fixed and adjustments modulated by the user using an external controller. The specifics of such learning circuits could be as simple as offset enhancement based on the difference between cardiac instruction and sensor reading, or much more complicated neural networks.

The light stimulator controller could be operated using an active latching system, a simple passive raster control between light emitters or an oscillatory circuit controlling each light emissive device. Pulse widths can be from 10 microseconds to 100 milliseconds but are most ideal in the couple of millisecond range. Thus, he most convenient way to control the LED’s is then by supplying a constant current which is pulsed with time. Latching circuits would turn on the signal and keep it on until a new signal would switch it off. Passive circuits would act to drive the circuit only when a drive signal is present. It may however be useful to also modulate the drive voltage and current. In the latter case, oscillatory circuitry would need to be designed accordingly.

If the oscillator is developed using analog circuitry, the capacitor required to achieve 1Hz may need to be external to the chip. Alternatively it can be created on-chip, but will require a large surface area. The chip may have a communication component which is connected to an RF or IR emitter/recorder. Both RF and IR signals can permeate the skin, and are thus the most
ideal transmission methods. This component may also be used to extract power from the external signal in order to recharge the battery. The communication bandwidth may be limited, and the energy cost of return transmission from the device could be significant. Thus pre-processing of the return signal from the chip would be important. Such a link would allow the user to modify the system to fine-tune the signalling, and to extract data where necessary.

Casing
The casing of the control unit functions as housing for the battery, electronics and light sources. The case will be made from a material such as titanium, a metal that is ten times as strong as steel, but much lighter. Titanium and two of its alloys, niobium and tantalum, are biocompatible and they exhibit physical and mechanical properties superior to many other metals. The modulus of elasticity (measure of stiffness) of titanium and its alloys range between 100-120GPa. A titanium case helps to shield the internal components and reduce the external electromagnetic interference. In addition, titanium casing shields from ground level cosmic radiation. Furthermore, while titanium is weakly paramagnetic, it is nevertheless considered MRI compatible.

Implantation
The control unit may be implanted as for present neurostimulator technology, for example subcutaneously. This may be connected to the active stimulator unit via optic fibre and optionally electrical control lines. The stimulator unit may be implanted as for present deep tissue neurostimulator technology except that care will be taken to allow axon outgrowth from the implant to connect to the target neurons or to innervate the target muscles. This can potentially be carried out with keyhole surgery.

In some cases, the optogenetic stem cell implant could be implanted by open surgery. Video assisted surgery (VATS) procedures are keyhole surgical procedures into the chest. They may be done as day-case surgery and require a short general anaesthetic. Using this procedure, it is possible to access the phrenic nerves for implantation of the stem cells and light delivery guide. Optic fibres may then be passed through the thoracic cavity or tunnelled under the skin to the control device.

A transcutaneous needle approach may be used to implant structures closer to the surface such as in the case of a parotid implant.

The control unit may be implanted as for present neuroprosthetic and pacemaker technologies. Implantation of the light delivery device and stem cells would ideally occur simultaneously to minimise procedures and to ensure accurate connectivity. This can be achieved in several ways.

Similar forms of keyhole surgery can be used for other implementations
A transcutaneous needle approach may be used to access the target nerves or muscle using ultrasound guidance and fine-bore needles. This technique would require local anaesthetic only and would allow delivery of stem cells or stem cell cage, which could then be stimulated transcutaneously.

**Applications**

Phrenic nerve degeneration The phrenic motor nerve controls contractions of the diaphragm, a key respiratory muscle in mammals, including human. Patients suffering from 'Amyotrophic Lateral Sclerosis' (ALS) loose the ability to breathe, because the disease destroys their phrenic motor neurons and thereby disconnects the diaphragm muscles from the neural circuit that provides it with breathing patterns. The *resuming* respiratory failure is the main cause for death from ALS. The neural implant described herein could be used to re-establish nerve-muscle connectivity between phrenic nerve and diaphragm and thereby rescue respiratory motor function.

Pharyneal nerve degeneration Patients suffering from ALS loose multiple motor functions due to motor neuron degeneration. One of the common symptoms, other than respiratory failure, is the loss of pharynx muscle innervation. As a result, the patients are no longer able to swallow and feed normally. Using an approach similar to the one we propose for phrenic nerve regeneration, the optogenetic neural implant could be used to regenerate the branchiomotor portions of the glosso-phyaryngeal and vagus nerves which supply pharynx musculature.

Regulation of visceral motor function Visceral motor functions, such as heart rate and blood pressure, are regulated by the sympathetic and parasympathetic neural circuits, the two parts of the autonomic motor system. These two sets of neural circuits often act in an antagonistic fashion. By grafting optogenetic neural implants into autonomic motor nerves, such as the parasympathetic cardiac branch of the vagus nerve, we could artificially control visceral motor functions in conditions where they are deregulated, such as tachycardia during myocardial infarction.

Optic nerve degeneration Sight is one of the most important sensory functions for human beings. Loss of vision is therefore highly significant to the individual. Glaucoma is one of the major causes of visual loss in the western world. It causes degeneration of the optic nerve cutting communication between the light sensing photoreceptors and the brain. Stem cell therapies to regenerate the nerve suffers from connectivity issues, opening up the possibility for optogenetic stem cell prosthesis.

Cardiac myocyte degeneration Electronic heart pacemakers and intracardiac defibrillators, though effective, are associated with many complications such as pacemaker failure, infection, venous thrombosis and lead dislodgement. Optogenetic approaches have been proposed, but optogenetic stem cell prosthesis could provide
significant advantage in forming new sino-atrial node connectivity, without worry about viral efficacy.
Other applications include, spinal cord prosthesis, bladder control prosthesis, erectile function prosthesis, peripheral nerve stimulation for those with facial and other palsies, parotid nerve prostheses.

Further Applications
The invention further relates to methods of medical treatment comprising implanting a population of cells as described above and/or a device as described above.

For example the invention relates to a method of treating Amyotrophic Lateral Sclerosis (ALS) in a subject comprising implanting a population of cells as described above into said subject. The invention further relates to a method of treating Amyotrophic Lateral Sclerosis (ALS) in a subject comprising implanting a device as described above into said subject.

In another embodiment, the invention relates to a method of treating a subject comprising collecting cells from said subject, extracting stem cells from said cells or inducing stem cells (such as iPS) from said collected cells; introducing a photosensor polypeptide of the invention into said cells; inducing differentiation of said cells; selecting motor neurons from said differentiated cells and reintroducing said motor neurons comprising said photosensor polypeptide into said subject. Optionally said method further comprises selecting astrocytes from the differentiated stem cells, and combining said astrocytes with said motor neurons before reintroducing same to said subject. Optionally said method further comprises placing said motor neurons, suitably together with said astrocytes, into a device as described above, and subsequently implanting said device into said subject.

The populations of cells described herein find industrial application in the treatment of disorders of motor neuron function. Such disorders include Amyotrophic Lateral Sclerosis (ALS). Such populations of cells may enjoy a separate life in commerce as saleable items, or may be used in the specific treatment of specific subjects, or may be used in the manufacture of medicaments such as in the manufacture of implantable device(s) as described above.

The invention finds application in the repair of muscular innervation via motor neurons such as repair of the phrenic nerve. In the context of the invention 'repair' does not necessarily imply reconnection to existing nerves in the subject but may instead
comprise providing a method of stimulating or inhibiting the target muscle(s) or nerve(s) directly via photostimulation or photoinhibition according to the invention.

**Brief Description of the Figures**

Figure 1 shows Chr2-expressing ESC-derived motor neurons can be induced to fire action potentials by a light stimulus. (A) ES cell-derived motor neurons carrying a doxycyclin-inducible Chr2-YFP excitatory photosensor gene were sorted magnetically from embryoid bodies using the Hb9::hCD2-GFP transgene, induced to express Chr2-YFP and cultured for 24h. (B) The cells were then stimulated with 500ms blue light pulses, and membrane currents/potentials were recorded by patch clamp. The light stimulus induces an inward current, which is sufficient to depolarize the motor neurons above threshold and trigger an action potential, is1/2 labels the nuclei of motor neurons. The blue bar under the traces indicated the timing and length of the light stimulus.

Figure 2 shows: ESC-derived motor neurons connect to muscle and control muscle contractions in vitro. EBs containing Chr2-YFP expressing ESC-derived motor neurons were co-cultured with muscle satellite cell-derived myofibres for 4 days. (A) The EB/myofibre co-cultures were then fixed and stained to detect postsynaptic nicotinic acetylcholine receptors on the muscle (aBTX), Chr2-YFP and axons (Nfr). Motor axons form close, synapse-like contacts with the muscle fibres. The dotted white lines mark the outline of a myofibre. (B) Innervated myofibres in live cultures were exposed to light pulses (10 Hz), and muscle contractions were recorded with a high-speed digital video camera. The red line marks the section of the myofibre which we analysed frame-by-frame. (C) The displacement caused by muscle contractions were quantified using the Mathlab software. The blue bar indicates the time period during which the culture was exposed to the light pulses.

Figure 3 shows Design of the optogenetic phrenic nerve neural implant. The scheme on the left shows the normal anatomy of the thoracic cavity and the trajectory of the phrenic nerve as it projects to the diaphragm muscle. The scheme on the right depicts the neural implant that we plan to use in order to restore diaphragm function in ALS, after degeneration of the phrenic nucleus. NMJ: neuromuscular junction.

Figure 4 shows an overview of the entire system:

<table>
<thead>
<tr>
<th>Control unit</th>
<th>[8] Light coupling connectors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>interface</td>
</tr>
</tbody>
</table>
The entire system with comprising of the subcutaneous module [1-11] described in more detail in other figures herein, and cabling [12] to the interface units [13,14] which are described in more detail in other figures herein. In summary, the processing unit [2] receives data from sensing units [14] and preprocessing components [4] which may be at the subcutaneous module, the interface unit or both. The processor signals the optical driver [3] to define the required light stimulation waveforms to be emitted from the light emissive module [7]. A cooling system [6] is used to remove excessive thermal energy from the light emissive module, the electronics or both. It can be fixed to the light emissive module, or the electronics module and connected to the unit casing for optimal thermal dissipation. The light from the array is coupled via optic guides [9] to the interfacing units (for example units [14]) via the main cable [12] this cable consists of optic fibres for the optical stimulation and sensing, and electrical connectors for electrical sensor and power. The functionality of the device can be monitored and changed via a RF/magnetic communication link [5].

Figure 5: Control unit.
[1] Battery or power source
[2] Communication system, e.g. RF or IR
[4] The power extraction component
[5] Communication decoding/encoding component
[6] Learning algorithm/neural network block
[7] Main processing block
[8] Memory block
[9] ECG signal acquisition and pre-processing
[10] Electronic sensor acquisition and pre-processing
[11] Optical sensor driver, data acquisition and pre-processing module
Cardiac photomodulation driver

Sensor optical system

Light emitters for cardiac photomodulation

This figure concentrates on the detail of the control electronics [3-12], power source [1], communication link [2], and optical subsystem [13,14]. The power source [1] could be a battery, or other charge storage system and possibly an additional energy scavenging system. The communication device [2] could be an RF coil or infrared optical system including emitters and receivers. [3] Represents the electronic components which can be integrated into a monolithic electronic chip. The memory storage system [8] could also be part of this chip, but may be better kept external. [4] Represents circuitry to extract power from the communication system. [5] [5] Represents the communication decoding/encoding component. [7] represents the main processing block which takes signals from the ECG [9] electronic sensor [10] and optical sensor [11] pre-processing units. If it determines to intervene and how to intervene it will signal action to the cardiac photomodulation driver[12]. It can use learning circuitry[6] and memory [8] to help in the final decision. Optical signals are then driven out via the photomodulation light emitters for cardiac stimulation/inhibition [14] and the emitters for the optical sensing systems [13]. On the return path, light will return via the sensor optical system [13] to optical sensors either externally in that system or inbuilt into the sensor circuitry [11].

Figure 6: Interfacing units

[1] Optic fibre enclosed with sheath to ensure structural stability and biocompatibility

[2] Optical connector to enclosure

[3] The stem cell enclosure, with waveguiding properties to ensure light delivery throughout to the differentiated stem cells

[4] Stimulating, recording electrodes

[5] Biosensors e.g. ISFET sensors

[6] Differentiated stem cells which have been photosensitized to express light sensitive channels, pumps or cascade systems

[7] Clamp to hold the system in place

This figure describes the interfacing units that will clip onto the desired destinations in the heart, as described in less detail in earlier figures.
Basic interfacing unit. Stimulating light is fed via an optic fibre in cable (a) and focus/imaged on the target tissue with micro-lens (e). An extracellular electrode (d) records the ECG signal and feeds it back to the control unit via cable (a). The electrode and the optic fibre tip are held together using a small chip (b) that is placed on the heart via clamping facilities (c).

Penetrating interfacing unit. Like [1] just that here the light guide is designed to penetrated inside tissue layer (e) to overcome opaque tissues.

Stand-alone interfacing unit. Like [1] with the exception that the light source (e) is built into the unit. It is driven by a flip-chip module (g) with/without internal battery.

Penetrating & transfecting interfacing unit. Like [2] with penetrating optics (e) just that here a transfected module is integrated. It includes a micro-reservoir to store the transfection reagents, control electronics (f) and a micro-needle (h) to direct the transfection.


Figure 7: Advance interfacing unit

[1] Light emissive module
[2] Surface modified sensor/MEMS component
[3] Surface electrodes for connecting to external sensor units and power supply
[4] Surface patterned micro-optics
[5] Surface patterned optical filter
[6] Transparent dielectric layers of CMOS chip
[7] CMOS silicon base
[8] Photodiode
[9] Processing elements
[10] Flip chip interconnects

This figure describes the potential physical arrangement whereby a monolithic CMOS chip integrates all the processing components and is bonded in a clip-chip or other arrangement with the light emissive elements. This will allow for miniaturized operation. [1] Describes the light emissive module, e.g. a light emitting diode array chip which is bonded to a CMOS chip [6,7] via interconnecting wires[10]. The CMOS chip typically has a semiconductor base [7] which can include circuits [9] and photodiode [8] sensors. If also has a transparent dielectric stack [6] which can be used for interconnecting wires, which can come to the surface to interface with the external
components of the chip [3], Surface modification such as MEMS sensors can be incorporated [2] in addition to optical filters [5] and microlenses [4]. In this arrangement a heat sink may be best placed at the back of the chip [11] or in a ring around the chip [12].

Figure 8: Driving circuit

[1] Information stream decoder and line and column control
[3] Control wires to LED (Which will sit on the LED chip)
[4] Memory unit and/or oscillator circuitry
[6] Active driver chip which is flip chip bonded to the GaN LED chip
[7] LED chip
[8] Control electronics for the passive unit

Figure 9 shows ES cells carrying a motor neuron-specific Mnx1::CD14-IRES-GFP BAC transgene were differentiated in vitro for 5 days, embryoid bodies were dissociated, and CD14+ motor neurons sorted by MACS. The different fractions from the sort were cultured for 1 day prior to IHC analysis. The purity of motor neurons in the eluate culture is ~95%.

Figure 10 shows ES cells carrying an astrocyte-specific GFAP::CD14 transgene were differentiated in vitro for 12 days, cell clusters were dissociated and CD14+ astrocytes were sorted by MACS. The different fractions from the sort were cultured for 3 days prior to IHC analysis. The purity of astrocytes in the eluate culture is ~80%.

Figure 11 shows ES cell-derived motor neurons and astrocytes were sorted by MACS as shown in figures 1 and 2. The motor neurons used in this experiment carry a Mnx1::CD14-IRES-GFP reporter transgene as well as a CAGGS::ChR2-YFP photosensor transgene. Following the sort, the two cell groups were combined into a population according to the invention in hanging drops, and astrocyte/motor neuron-aggregates plated at low density the next day. The two images show the live culture after 1 week.

This describes the potential driving circuit for the light emissive elements. There are two potential methods; active driving with individual oscillation control at each pixel [A] and passive control whereby elements are scanned in raster fashion [B]. In the active driver case signal information can be converted into the appropriate oscillation controls by line and column decoders [1]. These then send information to a memory...
unit or oscillator circuit [4] which determine whether or not current from the current source [2] passes through the LED power line [3] to ground [5]. The LED power line can protrude external to the chip to the light emitters for which a flip chip bonding process is highly appropriate [6]. The memory unit configuration [4] can be a simple latch which switches between on and off operation, whereas an oscillator [4] configuration would drive at a certain frequency until according to instruction. In the passive configuration [8] controllers can simply address a particular LED either on an individual basis or in an array. For the duration of the control current will pass through the light emitter allowing photons to be emitted.

The invention is now described by way of example. These examples are intended to be illustrative, and are not intended to limit the appended claims.

Examples

Example 1: Preparation of Motor Neurons

The protocol for the differentiation of motor neurons directly from stem cells such as mouse ES cells is essentially as described in "Directed differentiation of embryonic stem cells into motor neurons." Wichterle H, Lieberam I, Porter JA, Jessell TM. Cell. 2002 Aug 9;10(3):385-97. This document is expressly incorporated herein by reference specifically for the sections giving the protocol for differentiation of motor neurons.

Method is also disclosed in patent US 7,390,659 B2 (Columbia University). This document is expressly incorporated herein by reference specifically for the sections giving the protocol for differentiation of motor neurons.

When preparing motor neurons from human stem cells (such as human ES cells and/or human induced pluripotent stem cells) the method may advantageously be adapted as disclosed in "Differentiation of spinal motor neurons from pluripotent human stem cells." Hu BY, Zhang SC. Nat Protoc. 2009;4(9):1295-304. This document is expressly incorporated herein by reference specifically for the sections giving the protocol for differentiation of motor neurons.

It is especially advantageous to prepare cells of the invention from starting material from the subject into which the cells may ultimately be implanted. For example, induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. This is suitably performed as described in Dimos JT, Rodolfa KT,

Motor neurons generated according to any of the above protocols are then sorted (selected) as explained herein.

Example 1A: Provision of Optogenetic Function

The derived motor neurons of the invention suitably have a genetic modification.

The genetic modification required is the insertion of a molecular photosensor.

For example, the accompanying figures show mouse ES cell-derived motor neurons carrying a doxycycline-inducible channelrhodopsin-2 transgene.

For human applications, a human melanopsin transgene offers the advantage of lack of immune reaction. This also offers the advantage of signal amplification.

Example 2: Preparation of Companion Cells

In this example, the companion cells are astrocytes.

This example is only one method of many which may be used. This protocol has the advantage of providing a higher proportion of total cells. It also has the advantage of being faster (2.5 weeks instead of >3 weeks) than other methods.

Astrocyte differentiation from mouse ES cells:

1: ADFNK medium 2 days
On day 0, plate 2x10^5 dissociated ES cells per 10cm dish (Nunc Cat.No.263991), passage embryoid bodies (EBs) on day 1.

2: ADFNK medium + RA 3 days
Expand the EBs 1:4 onto 10cm dishes (Corning Cat.No. 430591) on day 2 in ADFNK + 1uM retinoic acid (RA) and expand again on day 4 1:2 in the same medium/dishes. It is possible to use ADFNK + RA + 0.5uM SAG (Merck) instead to direct the progenitor cells to a ventral spinal fate.

3: A1 medium 7 days
On day 5, plate EBs onto GFR-matrigel (BD) coated dishes in A1 medium. Dissociate cells after 2-3 days with 0.25% trypsin (Invitrogen) and plate in A1 medium as before. Passage the cells again more on day 9-10.

4: A2 medium 1-2 days
On day 12, dissociate adherent cultures with 0.25% trypsin and plate onto GFR-matrigel coated dishes in A2 medium.

5: A3 medium 5 days
On day 13-14, dissociate adherent cultures with 0.25% trypsin (Invitrogen).
Plate onto GFR-matrigel coated coversips/dishes in A3 medium. Astrocytes can be harvested (or fixed for immunofluorescent analysis) on day 18-19.

Media:

ADFNK

45% Advanced DMEM/F 12 medium
45% Neurobasal medium

10% Knock-out Serum Replacement (Invitrogen)
2 mM L-glutamine
1 X Pen/Step
beta-mercaptoethanol (1:1300 from 1:100 stock in PBS)

A1
Neurobasal medium
1 X B27 supplement
2 mM L-glutamine
1 X Pen/Step

10% ADFNK
10 ng/mL FGF-2
20 ng/mL EGF
10 ng/mL CTNF

A2
Neurobasal medium
1 X B27 supplement
2 mM L-glutamine
1 X Pen/Step

10% ADFNK
10 ng/mL FGF-2
20 ng/mL EGF
50 ng/mL BMP2

A3
Neurobasal medium
1 X B27 supplement
2 mM L-glutamine
1 X Pen/Step

10% ADFNK
10 ng/mL FGF-2
20 ng/mL EGF
Astrocyte differentiation from human ES cells:

For human ES cells, the differentiation may take longer. Thus this step should be extended to permit sufficient differentiation.

Example 3: Derivation of further companion cells

Oligodendrocytes can be generated from ES cells as described in Brustle et al. (1999 Science vol 285 p754). Oligodendrocytes will myelinate the motor axons inside the containment device, as they would normally do for the central portion of motor axons in the spinal cord.

In order to purify (sort/select) oligodendrocytes or their progenitors from ES cell differentiation cultures, surface antigens that can be used are those characteristic of oligodendrocytes as disclosed in "Enrichment of rat oligodendrocyte progenitor cells by magnetic cell sorting." Cizkova D, Cizek M, Nagyova M, Slovinska L, Novotna I, Jergova S, Radonak J, Hlucilova J, Vanicky I. J Neurosci Methods. 2009 Oct 30;184(1):88-94. This document is expressly incorporated herein by reference specifically for the disclosure of oligodendrocyte surface antigens.

Local spinal interneurons may be included in the cell population of the invention. The various interneuron subtypes are molecularly well-characterized and develop under the motor neuron-differentiation conditions.

In order to purify (sort/select) various interneuron subtypes the approach is as for motor neurons but selecting using appropriate surface antigens characteristic for the desired neuronal subtypes as in "Specification of neuronal fates in the ventral neural tube." Briscoe J, Ericson J. Curr Opin Neurobiol. 2001 Feb;1(1):43-9. This document is expressly incorporated herein by reference specifically for the disclosure of neuronal subtype specific surface antigens.

Example 4: Manipulation of Cell Population

The cell population may be advantageously aggregated. This may be accomplished by preparing the population as a 'hanging drop' culture.


There are numerous later papers describing essentially the same technique. For example, hanging drops can be used to aggregate cells into bodies: Efficiency of

Combining neurons and glia of the cell population of the invention is advantageously carried out according to this hanging drop technique.

A typical ratio of neurons:astrocytes is 1:10.

The volume per drop is typically 10-20µl.

Example 5: Dissociation of cells for sorting

It is advantageous to dissociate cells whilst retaining their surface antigens. This leads to efficient sorting using the surface antigens such as CD2 or CD14.

Advantageously the protease mixture used to dissociate the cells is selected to retain their CDs.

For the dissociation of Hb9::CD2GFP transgenic EBs (and subsequent MACS-enrichment of ES cell-derived motor neurons), we use Liberase DL (Roche). This reagent is a mix of collagenases I + II and dispase. It has the advantage of not cleaving CD2.

When CD14 is the sorting antigen, other more efficient proteases, may be used such as Liberase TH (Roche), Liberase TL (Roche), accutase (PAA) and/or 0.25% trypsin (Invitrogen).

All proteases are suitably used in combination with DNase I (Roche).

Example 6: Application of the Invention

The photosensor such as Chr protein allows the motor neurons to convert the light pulses into electrical activity, relay the signal to the diaphragm muscle and control cause it to contract rhythmically. The design of the optogenetic neural implant is shown in Figure 1. Chr2-expressing ESC-derived motor neurons are especially suitable. These neurons can be triggered to fire action potential by light pulses, and, when co-cultured with myofibres, connect with them and impose a light-controlled contraction rhythm.
With reference to figure 1, Chr2-expressing ESC-derived motor neurons can be induced to fire action potentials by a light stimulus, the following exemplary sequence was used:

**Sequence Chr2-H134R-YFP:**

```
ATGGACTATGGCGGCGCTTTGTCTGCCGTCGGACGCGAACTTTTGTTCGTTfiCTAATCCTGTGGTGGTGAACGGGTCCGTCCTGGTCCCTGAGGATCAATGTTACTGTGCCGGATGGATTGAATCTCGCGGCACGAACGGCGCTCAGACCGCGTCAAATGTCCTGCAGTGGCTTGCAGCAGGATTCAGCATTTTGCTGCTGATGTTCTATGCCTACCAAACCTGGAAA
```

The experiments shown in Figures 1 and 2 were done with motor neurons derived from mouse ES cells according to the present invention.

**Example 7: Exemplary Device**

The components of the photonic pacemaker may be:

1. Control unit containing
   a. Battery
   b. Processing
   c. Driving/control circuit
   d. Light source
   e. Optics with light guides
   f. Sensing components
   g. Communication link
   h. Casing
   i. Fixation facilities

2. Interfacing Unit containing
   a. Optics
   b. ECG sensing electrode
   c. Alternative modality sensors
   d. Transfection module
   e. Light source with power supply and driving circuit (optional)

Implantation of a functional photonic pacemaker may require the following steps (steps may be combined):

1. Confirmation of diagnosis
2. Selection of required stimulation or inhibition points
3. Preparation of transfection solution
4. Transfection of light sensitive gene
5. Implantation of control unit
6. Implantation of interfacing unit(s)
7. Programming of control unit
8. Testing

Example 8: Alternate Preparation of Motor Neurons

In this example the culture and isolation of mouse ES cell-derived motor neurons is demonstrated. The same technique can be applied to human cells.

- Grow ES cells (such as MnX1::hCD 14-ires-GFP clone# 13) in 24 well-plate on feeders until semi-confluent
- Dissociate ES cell colonies with trypsin (Invitrogen, Cat.No. 25200056)
- Plate 5x10^5 ES cells onto 10cm dish (Nunc, Cat.No. 150350).
- Passage EBs at day 1:1 in ADFNK
- Expand EB cultures at day 2 in 1:4 differentiation medium protocol (ADFNK medium, 1μM retinoic acid (Sigma-Aldrich, Cat.No. R2625-50MG), 0.5μM
- Smoothened Agonist, Cat.No. 566660-1 MG). Use 10cm dishes for suspension cells at this step (Corning, Cat.No. 430591).
- On day 4, passage EB 1:1 in differentiation medium

- Collect EBs on day 2+3 by sedimentation in a 15ml Falcon tube. Use at least 6x 10cm dishes.
- Wash them once with 10ml L15
- Add 2-4ml AccuMAX solution (Millipore, Cat.No. SCR006)
- 10min 37C in water bath, swirl every 2-3min
- Dissociate by pipetting 20x with P1000
- 5min 37C in water bath, dissociate again by pipetting 20x with P1000
- Add 5ml L15
- Check with microscope for single cell suspension
- Centrifuge 4min 300g
- Wash 3x with 8ml ADFN, 1:2000 DNasel (Roche, Cat.No. 04716728001)
- Take up in 1ml MACS buffer (D-PBS w/o Ca^2+, 0.5% sterile BSA, 1:2000 DNasel)
- Pass through 40μm Nylon cell strainer (BD Falcon, Cat.No. 352340), wash strainer with 1ml MACS buffer
- Centrifuge 4min 300g, fake dissociated cells in 3ml of MACS buffer
- Centrifuge 4min 300g, fake up in 200μl MACS buffer, add 1:100 a-hCD 14 (26ic)
- Take up in 500μl MACS buffer (at this point, samples for FACS and pre-MACS tissue cultures may be taken), count cell number
- Insert MS-column (Milteny) into MiniMACS magnet
- Pre-wash column with 500μl MACS buffer
- Apply the cell suspension to the column, collect flow thru in 15ml tube (Do not load more than 10^7 cells per MS column)
- Wash column 3x with 500μl MACS buffer
- Remove column from magnet, place on top of 15ml tube
- Elute cells from column with 1ml MACS buffer, count cell number in eluate. (The yield should be 30-40% of input cells)
Optional: Pipette 5x with P1000, pass through 40μm Nylon cell strainer, wash strainer with 1ml MACS buffer
- Add 10ml ADFNK medium, centrifuge 4min 300g
- Take up in culture medium (ADFNK medium, 10μg/ml GDNF), plate out on GFR-matrigel coated plates (Nunc Lab-Tek, Glass-CC2 or Permanox) at density of ca. 5x10^5/ml.
- Alternative: Aggregate neurospheres O/N in 20μl medium (ADFNK, 50ng/ml GDNF) on lid over 10cm dish filled with 10ml PBS. Use 5000 cells per drop.
- Monitor purity: pipette small aliquots of pre-MACS, flow-through and eluate into U-bottom 96 well plate, then take bright-field and green fluorescence image with inverted scope.

Media Used:

**ES medium 500ml**

400ml DMEM
75ml FBS (ES-cell certified)
20ml 4mix (5ml Pen/Strep, 5ml NEAA, 5ml nucleosides, 5ml L-Glutamine)
360μl L-mercaptoethanol  (1/100 diluted in PBS), 0.1 mM final
1000μl LIF supernatant (COS7 cells transfected with pCAGGS::LIF plasmid)

**ADFNK (AK) 500ml**

250ml Advanced DMEM-F12
250ml Neurobasal medium
56ml Knockout Serum Replacement
5ml Pen/Strep
3.75ml 200mM L-Glutamine
360μl L-mercaptoethanol (1/100 diluted in PBS), 0.1 mM final

**Example 9: Alternative Protocol for Astrocyte Differentiation From ES Cells**

In this example the ES cells are mouse ES cells. Human cells could also be used.

In this protocol, advantageously the embryoid bodies are plated. Since these are adherent, then minimal media can be used. This has the advantage of avoiding some trophic factors which can be required for growth in fluid. This is better. This is cheaper (by avoiding or reducing the amount of recombinant protein factors required).

1: ADFNK medium 2 days
On day 0, plate 5x10^5 dissociated ES cells per 10cm dish (Nunc Cat.No.263991), passage embryoid bodies (EBs) on day 1.

2: ADFNK medium + RA 3 days
Expand the EBs 1:4 onto 10cm dishes (Corning Cat.No. 430591) on day 2 in ADFNK + 1μM retinoic acid (RA) and expand again on day 4:1:2 in the same medium/dishes. It is possible to use ADFNK + RA + 0.5μM SAG (Merck) instead to direct the progenitor cells to a ventral spinal fate.
3: ADFNK medium 7 days
On day 5, plate EBs onto GFR-matrigel (BD) coated dishes in ADFNK medium. Feed the cells with ADFNK medium every 2-3 days. On day 12, dissociate the cells with Accumax (Millipore, Cat'.No. SCR006) and proceed with MACS purification.

Example 10: Cell Populations

Motor neurones comprising photosensitive transgene are produced as described above.

Magnetically purified (using CD14 surface tag as marker) motor neurons are shown in Figure 9.

Companion cells, in this example astrocytes, are produced as described above.

Magnetically purified astrocytes (using CD14 surface tag as marker) are shown in Figure 10.

These two cell types are combined into a population of cells according to the present invention. In this example, they are combined in hanging drops followed by culture in a 2D-format. The resulting population is shown in Figure 11.

These cell populations can be plated out at low density on a regular tissue culture dish. They advantageously remain stable.

In stark contrast to prior art cultures of pure motor neurons, populations of cells according to the present invention comprising motor neurons in the presence of astrocytes survive well and mature morphologically.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described aspects and embodiments of the present invention will be apparent to those skilled in the art without departing from the scope of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the art are intended to be within the scope of the following claims.
CLAIMS

1. An in vitro population of cells comprising
   (i) a motor neuron comprising a transgene; said transgene encoding a photosensitive protein capable of triggering or suppressing an action potential upon illumination with light of cognate wavelength; and
   (ii) an astrocyte.

2. An in vitro population of cells according to claim 1 wherein the motor neuron and astrocyte are each derived from stem cell (s).

3. An in vitro population of cells according to claim 1 or claim 2 wherein the photosensitive protein comprises channelrhodopsin (ChR) or melanopsin or halorhodopsin (NpHR).

4. An in vitro population of cells according to claim 3 wherein the photosensitive protein comprises channelrhodopsin (ChR) or melanopsin and wherein stimulation of said photosensitive protein triggers depolarisation of the motor neuron.

5. An in vitro population of cells according to claim 3 wherein the photosensitive protein comprises halorhodopsin (NpHR) and wherein stimulation of said photosensitive protein triggers hyperpolarisation of the motor neuron.

6. An in vitro population of cells according to any preceding claim wherein said population further comprises an oligodendrocyte.

7. An in vitro population of cells according to any preceding claim wherein said population comprises 50 to 5000 motor neurons.

8. An in vitro population of cells according to claim 7 wherein said population comprises 500 to 50,000 astrocytes.

9. An in vitro population of cells according to any preceding claim wherein said population comprises motor neurons and astrocytes in the ratio 1:10.

10. An in vitro population of cells according to any preceding claim wherein said cell(s) further comprise a second transgene encoding a genetic marker.
11. A population of cells according to any of claims 1 to 10 for use as a medicament.

12. Use of a population of cells according to any of claims 1 to 10 for the manufacture of a medicament for Amyotrophic Lateral Sclerosis (ALS).

13. A population of cells according to any of claims 1 to 10 for use in the treatment of Amyotrophic Lateral Sclerosis (ALS).

14. An implantable device comprising a population of cells according to any of claims 1 to 10, wherein said device is structured to retain the cell bodies within the device.

15. An implantable device according to claim 14 wherein said device comprises optically conductive material.

16. An implantable device according to claim 15 wherein said optically conductive material has a high refractive index.

17. An implantable device according to claim 15 or claim 16 wherein said device is formed as a light guide to conduct light from an input to the retained cell bodies.

18. An implantable device according to any of claims 15 to 17 wherein the device comprises pores to allow extension of axons outside the device.

19. An implantable device according to any of claims 15 to 18 further comprising an anchor.

20. A method of treating Amyotrophic Lateral Sclerosis (ALS) in a subject comprising implanting a population of cells according to any of claims 1 to 10 into said subject.

21. A method of treating Amyotrophic Lateral Sclerosis (ALS) in a subject comprising implanting a device according to any of claims 14 to 19 into said subject.

22. A population of cells or a device substantially as described herein.
Figure 8

(A): (Active Driver chip)

(B): (passive Driver chip)

Figure 9

<table>
<thead>
<tr>
<th>Mnx1::CD14-IRES-GFP DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-MACS</td>
</tr>
</tbody>
</table>

![Images of DNA distributions for pre-MACS, flow-through, and eluate samples.](image-url)
Figure 10

<table>
<thead>
<tr>
<th>GFAP::CD14 Gfap DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-MACS</td>
</tr>
<tr>
<td>flow-through</td>
</tr>
<tr>
<td>eluate</td>
</tr>
</tbody>
</table>

Figure 11

<table>
<thead>
<tr>
<th>bright field</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP/YFP</td>
</tr>
</tbody>
</table>