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(54) **Title**: METHODS, APPARATUSES, AND KITS FOR INTRODUCING GENETIC MATERIAL INTO LIVING CELLS

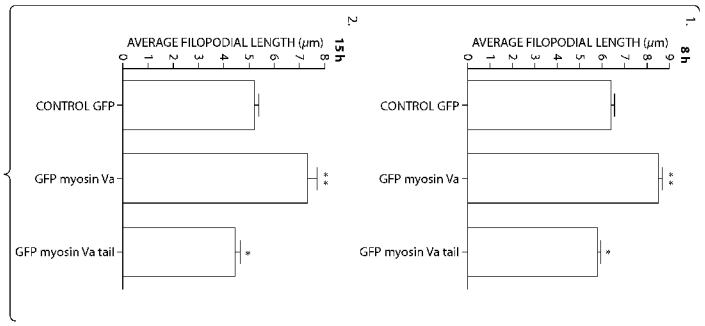


Fig. 4C

(57) **Abstract**: Methods, kits and devices are provided for introducing genetic material into cells, including post-mitotic types of cells such as neurons and sperm cells. The methods and devices utilize high fluid velocities, and cell survival following treatment by the methods including the loading process is enhanced compared to that of other transfection methods. The methods result in reliable highly efficient and rapid expression of recombinant proteins in the recipient cells.

Methods, apparatuses, and kits for introducing genetic material into living cells

Related application

The present international application claims the benefit of U.S. provisional application serial number 61/159,856 filed March 13, 2009 entitled "Methods, apparatuses, and kits for introducing genetic material into living cells", by inventor Thomas Diefenbach, which is hereby incorporated herein by reference in its entirety.

Technical field

High velocity methods, apparatus and kits are provided for introduction and expression of genetic material into living cells.

Background

Induced expression of genetic material, for example DNA sequences, in living cells is the foundation for molecular genetics and molecular biology. In this process, genetic material encoding for example for a gene, is artificially introduced into the nucleus of a cell, so that the cell generates the product of the genes in the form of non-native or modified proteins, as is achieved by genetic engineering. In microbiology, the method by which one or more particular genes is altered in a recipient cell is referred to as transformation. In eukaryotic biology this method is sometimes referred to as transfection, or the introduction of a foreign cloned gene or cDNA into the eukaryotic genome. Viral or plasmid vectors are the vehicle for the introduced DNA sequences.

Many methods have been employed to introduce genetic material into the nucleus or cytoplasm of living cells. These methods have limitations and require the conveyance of plasmid cDNA across plasma membranes, which are the heterogeneous bilayers of lipid molecules found on all cells. A well-known method to introduce genetic material into the nucleus or cytoplasm of living cells is electroporation, which relies on dielectric breakdown of the membrane producing gaps of up to 120 nm in diameter (metastable aqueous pores) in the membrane through which genetic material enters the cell through electrodiffusion. Another method is the use of transfection reagents including lipid or fat-based reagents (i.e., lipofectamine) which are essentially detergents that associate with DNA, thereby permitting the DNA to pass though the plasma membrane.

Biolistic transfection is yet another method, and involves a "gene gun" that fires the genetic material coupled to gold nanoparticles at high pressure through the plasma membrane. Mechanical injection is yet another method that typically uses glass needles that physically puncture the plasma membrane to deliver the genetic material using hydrostatic pressure injection directly into the nucleus.

Efficiencies of these methods have been found to have limitations, particularly due to toxicity, injury or death to the cells. The transfection efficiency of such methods typically is very low or variable, depending upon the method and the cell type, and is related to the loss of viability of the treated cells, or the inability of the method to get the genetic material through the cell membrane. For example, transfection efficiency of the gene gun to treat a variety of different cell lines was observed to be successful with only about 1% to 4% of treated cells. Lipofection yields successful recombinants in only about 10% to 20% of treated cells. Polycationic lipid reagents generally do not exceed 40% efficiency. Most importantly, certain cell types are not amenable to any methods, including types of immune system cells, human stem cells, muscle cells, nerve cells, and other cell types that do not divide and are therefore maintained in culture only as primary cells.

A more biological approach relies on virus-mediated transfection or introduction of genetic material into cells. This approach utilizes specific engineered virus vectors derived from strains such as adenovirus, sindbis virus, retrovirus, baculovirus or lentivirus, etc. to infect the cells. The viral genomes are engineered to carry the genetic sequences of interest, and are consequently a relatively time-consuming and labor-intensive method compared to mechanical means of introducing genetic material. This approach has a significant methodological limitation in that the time delay or lag to obtain protein expression depends not only on the efficiency of the cell to transcribe and translate the genetic material, but also on the infection efficiency of the virus. In addition, use of live virus requires special laboratory safety standard conditions, i.e., biohazard level 2+.

Infection efficiency depends on many variables, and viral-mediated engineering is characterized by a lag of at least a day or several days to observe protein expression in cell populations. Furthermore, infection efficiency is cell-type specific, i.e., particular viruses do not infect certain cells.

There remains a need for a rapid, efficient, and universal method for introducing genetic material into any type of cell, including cell types that have heretofore been difficult or impossible to transfect with any commercially available method.

Summary

An aspect of the present invention provides a pressurized fluid flow apparatus including: a pipe portion having an inner diameter and an outer diameter and a distal end and a proximal end, such that the distal end is open to the atmosphere, such that the inner diameter of the pipe portion is about 1.0 mm to about 5.0 mm; the apparatus further including an exit tip adjacent to and contiguous with the distal end of the pipe portion, such that the exit tip has an inner diameter and an outer diameter, the inner diameter of the exit tip being about 0.1 mm to about 0.5 mm; and a suction device connected to the proximal end of the pipe portion for generating positive pressure and negative pressure.

A related embodiment of the apparatus further includes a receptacle adjacent to the exit tip for receiving and retaining fluid passaged through the pipe portion and exit tip. An embodiment of the apparatus includes a pipe portion with a diameter that varies along the length of the pipe portion, for example, from a larger diameter to a smaller diameter to a larger diameter, the smaller diameter constituting a constriction of the pipe.

An embodiment of the pipe portion and the exit tip includes at least one material selected from: borosilicate glass, aluminosilicate glass, and the like. In a related embodiment, the material includes flint glass (including lead oxide, titanium dioxide, zirconium dioxide or similar metals) and the like. Alternatively, the material includes a plastic, such as a polymer.

An embodiment of the apparatus includes the distal end of the exit tip polished by heat treatment to create a non-lacerative surface. For example, heat treatment includes flame-polishing. A non-lacerative surface of the exit tip minimizes shearing of cells when encountering the edge of the exit tip at high velocities, thereby improving efficiency of the process by increasing viability of treated cells.

In general, the suction device generates a flow velocity along the length of the pipe portion, the velocity selected from at least one of a group consisting of about: 5 cm/s, 15 cm/s, and 20 cm/s. In An embodiment of the apparatus, the suction device generates a flow velocity along the length of the pipe portion, the flow velocity selected from at least one of about 5 cm/s to about 10 cm/s, about 10 cm/s to about 15 cm/s, about 15 cm/s to about 20 cm/s, and about 20 cm/s to about 40 cm/s.

In general, the suction device generates a mass flow rate at the exit tip selected from at least one of about: 0.03 ml/min, 0.06 ml/min, 0.3 ml/min, 0.5 ml/min, 1.0 ml/min, and 2.0 ml/min. In An embodiment of the apparatus, the suction device generates a mass flow rate at the exit tip selected from at least about: 0.01 ml/min, 0.05 ml/min, 0.1 ml/min, 0.2 ml/min, 0.4 ml/min, 0.8 ml/min, 1.2 ml/min, 1.5 ml/min, and about 3 ml/min.

A related embodiment of the apparatus includes the suction device that generates a mass flow rate along the length of the pipe portion selected from at least one of about: 1.0 ml/min, 5 ml/min, 10 ml/min, 50 ml/min, 100 ml/min, and 150 ml/min. For example, the suction apparatus generates a mass flow rate along the pipe portion selected from at least one of about 15 ml/min, at least about 25 ml/min, at least about 40 ml/min, at least about 60 ml/min, at least about 75 ml/min, at least about 90 ml/min, or at least about 125 ml/min.

An embodiment of the suction device is manually controlled. The phrase "manually controlled" as used herein means operated directly by the user, so that at least one of the choices of flow rates and access to cells and receptacles is controlled by the user at the time of use. In an alternative embodiment, the suction device is automated, i.e., is at least partially or completely controlled automatically so that the user need not even be present, for example the device is robotic and operationally linked to a computer program and computer. A related embodiment of the suction device includes at least one device that is manually controlled, and at least one device that is automated.

An embodiment of the exit opening further includes a constriction in a continuous flow system controlled by at least one valve, for example, is controlled by two, three, or four valves, etc. For example, the at least one valve is a plurality of valves is positioned laterally proximal to the exit, within millimeters or micrometers proximal to the exit, or all are proximal to the exit, for example, arrayed circularly around the exit. Alternatively, at least one or more of the plurality of valves is distal to the exit.

An aspect of the present invention provides a method for introducing genetic material into a cell, the method including: contacting in a receptacle at least one cell with a composition including an effective amount of the genetic material to obtain a resulting mixture; and, inserting the apparatus of any of the embodiments herein into the receptacle and passaging the mixture of cells and genetic material through the apparatus at least once, such that passaging the mixture having the cells and the genetic material through the apparatus introduces the genetic material into the cell. In general, a composition includes a fluid. For example, the fluid includes a solution or a suspension.

In an embodiment of the method, the cell is a prokaryotic cell. Alternatively, the cell is a eukaryotic cell.

In general, introducing the genetic material is localizing the genetic material to the nucleus of the cell. Alternatively, introducing the genetic material is localizing the genetic material into the cytoplasmic or non-nuclear parts of the cell, for example, into the endoplasmic reticulum, Golgi apparatus, mitochrondrion, and/or lysosomes. In a related

embodiment, localizing further involves visualizing the genetic material in the nucleus or in the cytoplasm of the cell with a detectable marker. For example, the detectable marker is an agent that is at least one of fluorescent, colorimetric, enzymatic, radioactive, and the like.

An embodiment of the method includes passaging the mixture by dispensing into the receptacle. Alternatively, the method involves passaging the mixture through the apparatus, and dispensing the mixture into a plurality of receptacles. For examples, passaging further involves dispensing the mixture of the cell and the genetic material at least once into the same receptacle, i.e., the receptacle that originally housed the cells. Alternatively, the method involves passaging the mixture through the apparatus, and dispensing the mixture into each of a first receptacle and a second receptacle. An embodiment of the method involving a large number of receptacles is envisioned as automatically controlled, for example, by robotics.

An embodiment of the method includes passaging the cell and genetic material in the apparatus at least once for a period of time selected from at least one of about: 0.1 minutes, 0.3 minutes, 0.5 minutes, 0.75 minutes, 1 minute, 1.5 minutes, 2.0 minutes, 5.0 minutes, 7.0 minutes, 10.0 minutes, 15.0 minutes, 20.0 minutes, and 30 minutes.

In general, the cell is a member of a population in a plurality of cells. In general, the viability of the cells is not substantially reduced, i.e., the efficiency of plating of cells of the population remains substantially the same in comparison to control cells not passaged, or control cells passaged absent genetic material.

An embodiment of passaging includes dispensing the mixture of cell and the genetic material at least once into the receptacle, i.e., removing cells from the receptacle into the apparatus and distributing cells into the receptacle at least one once.

An embodiment of the receptacle is a centrifuge tube. An embodiment of the method includes passing or passaging the mixture of cells and genetic material through a constriction in a continuous pipe having an entry point and an exit point. Such a system is used, for example, in a flow-based, high throughput transfection system.

In general, the fluid includes a calcium concentration that is less than about 200 nM. For example, the composition includes a calcium concentration that is less than about 150 nM, less than about 100 nM, or less than about 50 nM.

An embodiment of the fluid includes a magnesium concentration that is at least about 1.5 mM. For example, the fluid includes a magnesium concentration of at least about 3 mM, at least about 10 mM, at least about 20 mM, or at least about 50 mM.

In related embodiments, the method further includes, after passaging, centrifuging the mixture to obtain a cell pellet and supernatant. For example, the method further includes

removing the supernatant, adding cell culture medium to the receptacle, and re-suspending the cell pellet in the medium. In a related embodiment, the method further includes culturing the cells.

In related embodiments of the method, the cells are living postmitotic cells. For example, the postmitotic cells are neurons or sperm cells. For example, the neurons are ciliary ganglion neurons or dorsal root ganglion neurons.

Alternatively, the cells are living premitotic cells. For example, the premitotic cells are at least one cell type selected from epithelial, hematopoietic, liver cells, and spleen cells.

In An embodiment of the method, the cells are physiologically inactive. In a related embodiment, the physiologically inactive cells are selected from: inhibited, UV-inactivated, enucleated, anucleate, and heat-killed.

In general, the genetic material is DNA or RNA. In a related embodiment, the DNA is cDNA. Alternatively, RNA is at least one selected from mRNA, tRNA, rRNA, siRNA, RNAi, miRNA, and dsRNA.

In an embodiment of the method, the fluid includes at least one transfection agent. In related embodiments, the at least one transfection agent is selected from: a nanoparticle, a liposome, a viral vector, a bacteriophage, and a detergent. For example, the transfection agent is Lipofectamine.

An embodiment of the invention provides a kit for introducing genetic material into a nucleus of a living cell, the kit including the apparatus of any of the embodiments herein.

In related embodiments, the kit further includes a receptacle, or instructions for use, or a transfection agent. For example, the transfection agent is selected from: a nanoparticle, a liposome, a viral vector, a bacteriophage, and a detergent. For example, the transfection agent is Lipofectamine.

An embodiment of the invention provides a method for introducing genetic material into at least one cell in a tissue or a monolayer of cells in culture, the method including: inserting the apparatus of any of the embodiments described herein into a receptacle, such that the receptacle contains a composition including an effective amount of the genetic material; and, contacting the at least one cell in the tissue or the monolayer in culture, such that contacting includes ejecting the composition under pressure onto the cell or cells, whereby the genetic material is introduced into the cell in the tissue or the cells of the monolayer.

In general, ejecting the composition under pressure includes generating a pressure wave having a particular frequency. The phrase "mechanical waves" refers to waves which

propagate through a material medium (solid, liquid, or gas) at a wave speed that depends on elastic and inertial properties of that medium. Wave motions of mechanical waves include longitudinal waves and transverse waves. In general in the methods herein, the pressure wave is a longitudinal wave, and the particle displacement is parallel to the direction of wave propagation.

An embodiment of the method further includes, following contacting, observing the genetic material entering the at least one cell without disrupting cell membranes or tissue. For example, observing includes analyzing cell membranes using a microscope. In an embodiment of the method, the cell is a prokaryotic cell. Alternatively, the cell is a eukaryotic cell.

In general, ejecting the cells from the apparatus results in localizing the genetic material to the nucleus of the cell. Alternatively, ejecting results in localizing the genetic material to cytoplasmic parts of the cell: e.g., endoplasmic reticulum, Golgi apparatus, mitochrondrion, and lysosome.

An embodiment of the method includes visualizing the genetic material with a detectable marker. In related embodiments, the detectable marker is an agent that is at least one of fluorescent, colorimetric, enzymatic, or radioactive.

In general, the cell is a plurality of cells. In an embodiment of the method, the cell is a living cell within a population of living cells, and the viability of the cells is not substantially reduced.

In general, the genetic material is DNA or RNA. For example, the DNA is cDNA. For example, the RNA is at least one class of RNA selected from the group consisting of mRNA, tRNA, rRNA, siRNA, RNAi, miRNA, and dsRNA.

In general, the tissue is a mammalian tissue. For example, the mammalian tissue is a human tissue, for example, skin, kidney, pancreas, liver, lung, heart, brain, spinal cord, bone marrow, and eye. An embodiment of the monolayer includes stem cells, for example, at least one stem cell selected from hematopoietic, hemangioblast, mesenchymal, hepatocyte, pancreatic, pulmonary, neural, fetal, and embryonic stem cells.

Brief description of the drawings

Fig. 1 is a set of photographs showing cells photographed under different detection conditions. The photograph on the left is a photograph of a brightfield microscope field of view showing a collection of ciliary ganglion neurons carrying a gene for green fluorescent protein (GFP) introduced by the methods and devices herein, photographed at time point

seven hours after culturing. The photograph on the right is a photograph of an epifluorescence microscope field of view of the same ciliary ganglion neurons photographed at the same point in time.

Fig. 2 panel A is a set of photographs each showing the same nonneuronal ciliary ganglion cell that is the recipient of a gene encoding green-fluorescent protein (GFP), introduced by the methods and devices herein. The photograph on the left is a brightfield microscope field of view, and the photograph on the right is an epifluorescence microscope field of view.

Fig. 2 Panel B is a set of photographs each showing a ciliary ganglion neuronal cell that is the recipient of genes encoding GFP, introduced by the methods and devices herein. The photograph on the top is a brightfield microscope field of view, and the photograph on the bottom is a fluorescence microscope field of view. These data show that GFP expression is maximal at 48 hours after receiving the GFP gene. Further, GFP expression is abundant in compartments in the cytoplasm and absent from the nucleus.

Fig. 3 panel A is a set of photographs each showing a ciliary ganglion neuron that is the recipient of a gene encoding a fusion of GFP-myosin Va, introduced by the methods and devices herein, photographed at time point eight hours after having the genetic material introduced into the cell. The photograph on the left is a brightfield microscope field of view, and the photograph on the right is an epifluorescence microscope field of view, each taken at the same point in time. The photographs of the cells treated with GFP-myosin Va by methods described herein show a specific distribution in the neuronal processes typical of native myosin protein.

Fig. 3 panel B is a set of photographs each showing the same ciliary ganglion neuron from the same culture as shown in Fig. 3 panel A, photographed at time point 28 hours after having the genetic material introduced into the cell. The photograph on the left is a brightfield microscope field of view, and the photograph on the right is an epifluorescence microscope field of view, each taken at the same point in time. These photographs show localization of myosin Va to each of the cell body and the neuronal processes, particularly the branch points (b) and the tips of the growing axonal processes (indicated in the immunofluorescent photographs by asterisks).

Fig. 4 is a set of photomicrographs and bar graphs showing effects entry of genetic material by methods described herein observed on fluorescence abundance of the tips of neuronal projections (growth cones), and filopodial length, respectively. Cells were transfected with a each of a construct carrying a gene encoding enhanced GFP-myosin Va

(myosin-Va GFP), an enhanced GFP-myosin Va that includes only the tail region (myosin-Va tail GFP) and therefore does not bind the actin cytoskeleton, or a control GFP construct (GFP). Cells were transfected and probed for fluorescence after 15 hours in cell culture. Each pair of images of a single cell includes at a brightfield (Nomarski or differential interference contrast microscopy, DIC) micrograph on the left and a matched fluorescence image of the same cell on the right.

Fig. 4 panel A is a set of fluorescence image photomicrographs taken with a laser scanning confocal microscope (LSCM) of neuronal growth cones transfected with constructs carrying a gene (myosin-Va GFP, myosin-Va tail GFP and GFP control). Each of a construct encoding an enhanced GFP-fusion protein encoding the full-length (FL) neuronal isoform of myosin-Va heavy chain chicken, and a construct encoding a truncated form consisting of the full tail (FT) region that includes the entire IQ motif, were transfected into neurons using a method described herein. Growth cones of cells transfected with the construct carrying the gene encoding myosin-Va GFP showed increased abundance of enhanced GFP myosin-Va in the central and peripheral growth cone regions and along neuronal projections compared to cells transfected with the construct carrying the gene encoding myosin-Va mutant tail region or a control GFP construct control. These data demonstrate successful transfection of neuronal cells with the two different myosin Va protein-encoding constructs, and also a role of myosin-Va in filopodial length extension. Scale bar is 10 μm.

Fig. 4 panel B is a set of brightfield DIC micrographs of individual growth cones transfected with a construct carrying a gene encoding myosin-Va GFP, a construct carrying a gene encoding myosin-Va tail GFP, or a construct carrying a gene encoding the GFP control. Growth cones transfected with the gene encoding myosin-Va GFP were observed to display significantly longer finger-like membrane projections (filopodia) than growth cones transfected with the control gene encoding GFP. Growth cones tranfected with the construct carrying the gene encoding the mutant tail region of myosin-Va showed significantly shorter filopodia compared to growth cones transfected with the gene encoding GFP or the gene encoding myosin-Va GFP. Data from these photomicrographs demonstrate that successful transfection was observed, and that transfection of the different myosin Va protein variants resulted in specific and different effects in growth of nerve cell projections at the growing tips. Scale bar is 10 μm.

Fig. 4 panel C is a bar graph showing filopodial length of growth cones observed at each of eight hours (h) and 15 hours after transfection and plating. Enhanced GFP-myosin-Va overexpression was observed to result in significantly increased average filopodial length at

eight hours ($p < 0.01$, Student's t-test) and 15 hours post-transfection. Transfection of enhanced GFP-Va tail was observed to result in a slight but significant reduction in filopodial length ($p<0.01$, Student's t-test) at 15 hour compared to eight hours. Number of filopodia/growth cones for each construct group include: control GFP (538/59), enhanced GFP-myosin-Va (630/69), and enhanced GFP-Va tail (653/76). The data show that overexpression of myosin-Va resulted in increased filopodial lengths, and expression of a truncated form of myosin-Va, which does not bind actin filaments, resulted in reduced filopodial lengths. These data show functional, successful transfection of myosin-Va constructs into primary neurons using the methods herein, and expression of differential functions of the encoded genes.

Fig. 5 is a set of pairs of photographs each pair showing untreated sperm cells (Untreated) on the left, and the same sperm cells that were recipients of genes encoding a variant of green fluorescent protein, the variant entitled pCherry (Shaner NC et al., 2004, Nat Biotechnol 22(12):1567-1572), introduced by the methods and devices herein (pCherry) on the right of each pair. The photographs on the left (a, c, and e) are fluorescence microscopy fields of view, and the photographs on the right (b, d, and f) are Nomarski or differential interference contrast microscopy fields of view.

The images of the left panel show the Untreated group of sperm cells. Fluorescence intensity is due to background autofluorescence (fluorescence native to the cell). Panels (a) and (b) show a low power field of view with many sperm cells. Bright fluorescence points are debris from other cell types. Panels (c) and (d) show a higher power field of view illustrating the background level of nonspecific fluorescence in untreated sperm cells from the same field as in (a) and (b). In the right panel of images (pCherry recipient) sperm cells treated with the methods of devices herein show expression of pCherry protein as evidenced by significant fluorescence throughout the cells especially in the sperm head and midpiece, with a lesser amount observed in the principle piece, or sperm tail. Panels (a) and (b) show a low power field of view of a group of treated (pCherry) sperm cells. Panels (c) to (f) show high power fields of different views of sperm cells with prominent pCherry fluorescence.

Detailed description of embodiments

The invention herein provides methods and devices for introducing genetic material into living cells. The methods and devices herein utilize high fluid velocities, and the examples herein show that cell survival during the loading process is enhanced, resulting in reliable highly efficient expression of recombinant proteins in cultured cells.

Thus, without being limited by any particular theory or mechanism of action, the methods herein using fluid dynamic technology, in combination with low calcium conditions, passages cells at such high velocity that plasma and possibly also nuclear membranes are stretched momentarily, thereby producing transient holes in the membranes that permit entry of plasmid DNA in a molecular shape that is long and relatively thin. In fact, the diameter of a single DNA helix is 2nm. A circular cDNA plasmid is subject to coiling and supercoiling, resulting in a structure that has a configuration capable of entering a cell through resulting openings in the cell membrane produced by the method. Given that electroporation results in openings as great as 120nm in diameter, it is possible that the methods herein result in openings in the membrane similar to these sizes.

Without being limited by any particular theory or mechanism of action, it is envisioned that the production of holes in the membrane of cells which cells are passing through a constriction in a fluid passageway occurs through the Bernoulli principle, the process by which an incompressible fluid moving into a region having a different cross-sectional area undergoes a change in speed. For the change in speed to occur, there must also be a change in force. A change in force correlates to observing a change in pressure, for example, an increase in speed results in a drop in pressure.

Cells moving in a fluid-filled channel or passageway undergo an increase in speed in a constriction of the channel, or in passing through a narrower diameter exit aperture. These cells in suspension experience a sudden change from high pressure to low pressure. Low pressure results in temporary stretching of the cells, creating temporary holes in cell membranes, thus permitting entry of genetic material present in the fluid or solution surrounding the cells, including without limitation exemplary genetic materials cDNA, siRNA, miRNA. Low calcium concentration in the fluid or solution promotes prolongation of cell membrane sealing, further promoting entry of genetic material down a pressure and concentration gradient through the holes in the membrane and into the cell interior. The Bernoulli principle is a mechanism by which fluid pressure facilitates entry of large molecules such as DNA into cells.

The technology provided herein is envisioned also as being suitable to be incorporated into automated robotic systems, for reliable introduction of DNA into cells in a rapid and high-throughput manner.

The cultured neurons used herein are postmitotic and therefore have reached a final developmental stage and do not further divide. Neurons are fragile cells, and prior art manipulation techniques, such as electroporation, have been found to be damaging to the

cells such that regeneration capabilities for physiological processes and viability in culture are negatively impacted.

Further, primary cultures of certain neurons such as ciliary ganglion neurons and dorsal root ganglion neurons, have typically been used at a stage that is optimal for experimental manipulation, from one hour to 12 hours after culturing. Beyond 12 hours after culturing, the neuronal processes become so intertwined and overgrown that even in low density cultures the ability to monitor neuronal regeneration and outgrowth becomes increasingly limited with time. Thus, a method to rapidly introduce and express genetic material in cells such as neurons remains a long-felt need. It is envisioned that cell-based experimental systems that require rapid expression of genes will benefit from the methods and apparati described herein. Furthermore, high-throughput transfection required in such processes as protein production can be improved using the rapid and efficient nature of this method and apparatus. Another application of this method is found in cell-based gene therapy for clinical use, for example, gene silencing using siRNA.

The methods herein for introducing genetic material into cells provide significant improvements over the prior art, for several reasons. The method is rapid, reliable, economical (there is no need for expensive reagents) and recipient cells show very high expression efficiency. Using electroporation, the highest efficacy obtained is only about 40% of recipient cells that express the nucleic acid. For adenovirus infection expression the highest efficacy obtained is about 30% to 85% and is generally lower. Gene guns achieve typically between 1% and 5% efficiency.

In contrast the methods herein result in visible protein expression in neurons (postmitotitic cells) within 3 hours post-transfection. In addition, the present method achieved consistently high transfection efficiencies of 80% to about 100% within 24 hours. Furthermore, the methods herein advantageously do not require viruses, for example, recombinant adenoviruses, which require biosafety level 2+ due to infectiousness. The present method is performed without use of toxic chemicals, complex procedures, or viruses. Furthermore, the methods herein deliver DNA to cells at the time that the cells are dissociated for plating and can therefore be applied to cells of a tissue that are obtained by dissociation, or can be applied to cell suspensions, e.g., primary cells, cell lines. Alternatively, the methods herein are applied to a tissue in situ, or to a monolayer of cells in culture. The methods herein bypass the relatively lengthy periods of time required for chemical or viral vectors to express genes for functional analysis (hours or days). Certain methods such as cationic reagents, the gene gun, and electroporation are limited by the size or length of cDNA

that can be used, thereby limiting the types of proteins which can be addressed in an experiment or screen. Examples herein include use of a very large plasmid encoding the full-length protein of myosin Va, which has a molecular weight of about110kDa. The data in the Examples herein show that constructs of different sizes, for example DNA encoding proteins that are much larger than myosin Va, were introduced into cells more effectively and efficiently using the method, apparatus and kits of the present application than by conventional methods. Introducing the genetic material in Examples herein also includes using a transfection agents or a plurality of transfection agents. For example, the transfection agent is a nanoparticle, a liposome, a viral vector, a bacteriophage, and a detergent. For example, the transfection agent is Lipofectamine.

An exemplary method includes one passage, or a plurality of passages, for example about five to ten passages, that are performed within a one minute period. For example, a single passage or multiple passages, repeated passages performed at more than one time, are rapidly performed. Depending on the cell type, additional embodiments are envisioned to include passaging five times every ten minutes for a specific period of time, for example, during a time period of about one hour, about two hours, or about five hours.

In general, it was consistently observed that a single passage was sufficient to introduce genetic material into a plurality of cells. Cells were maintained in cell culture medium at a suitable temperature, e.g., 37 °C, after a single treatment, or between treatments.

The term "introducing" herein refers to a variety of methods of delivering genetic material into a cell, either *in vitro* or *in vivo*, such methods including transformation, transduction, transfection, and infection. Vectors include plasmid vectors and viral vectors. Viral vectors include retroviral vectors, lentiviral vectors, or other vectors such as adenoviral vectors or adeno-associated vectors.

The term "passaging", as used herein means impelling a composition or a mixture, for example a solution or a suspension, through at least a portion of a length of the apparatus, i.e., a proximal portion, a middle portion, or a distal portion. In related embodiments, passaging means impelling the fluid entirely through the apparatus, i.e., removing a suspension of cells from a receptacle into the pipe portion and replacing the cells into the same receptacle or distributing the cells into a plurality of receptacles.

The method herein is performed on many different cells types some of which have been considered refractory to genetic manipulation in the past. Cell types include immortalized cells, actively growing cells, for example, cells in culture, and also postmitotic cells. Examples herein demonstrate successful use of the methods with cells that have not in

the past conveniently responded favorably to transfection reagents or to electroporation, resulting in low viability, or cell-type specific differences in transfection efficiency. Further, expression of protein encoded by the introduced genetic material using the methods herein was found to be significantly more rapid, within three hours of the treatment, which is at least about two to three fold faster rate of expression of genes that are introduced by prior art methods.

The myosin-Va constructs described in Examples herein are derived from the chicken neuronal isoform of myosin-Va (Espreafico E.M., et al., 1992 *J. Cell Biol.* 119(6): 1541-1557). A construct pCB6-FL (EGFP-MVa with FL referring to full length) contains the myosin-Va full length heavy chain (amino acid residues 1-1830) in a pCB6 plasmid. A truncated form of MVa consists of the entire last IQ motif (from ARV to SRV, amino acid residues 880-1830) pEGFP-FT (EGFP-MVaFT, with FT referring to full tail). These constructs expressed in melanoma cells were shown to have effects on melanosome trafficking (da Silva Bizario J.C., et al., (2002) *Cell Motil. Cytoskeleton* 51(2): 57-75).

The invention having now been fully described, it is further illustrated by the following examples and claims, which are illustrative and are not meant to be further limiting. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are within the scope of the present invention and claims. The contents of all references, including issued patents and published patent applications cited throughout this application, are hereby incorporated by reference.

Examples

Example 1: Deficiencies of prior art techniques

Methods to load antibodies into neuron cells (trituration) have been attempted to reduce protein expression (Beerman et al., (1994) Methods Cell Biol 44: 715-732; Buchstaller et al., (2000) Microsc Res Tech 48(2): 97-106; Diefenbach et al., (2002) J Cell Biol 158(7): 1207-1217; Diefenbach et al., In: Celis JE, Carter N, Simons K, Small JV, Hunter T, Shotton D, editors. (2002) Cell Biology-A Laboratory Handbook. 3rd Edition (4 volumes). As antibody molecules are significantly smaller in molecular mass and overall length than are sequences of plasmid DNA, and as a DNA molecule would have to pass through not only the plasma membrane but also the inner nuclear membrane, these prior

efforts have demonstrated the limitations of getting such larger macromolecules such as plasmid DNA into cells or into the nuclei of cells.

Trituration requires substantially higher velocities and changes in pressure for the significantly larger plasmid DNA to penetrate one or two membrane systems of the cell. Further, such higher velocities result in loss of membrane integrity, and indeed trituration loading at higher velocities causes substantial cell death, the creation of cellular debris from ruptured cells, introduction of air bubbles, and difficulties in suitable design and method.

Examples 2: Design of an apparatus to introduce genetic material into living cells

To produce a device to introduce genetic material into living cells, borosilicate glass pipettes were flame-polished at one end (tip) to reduce the size of the opening to less than or about 0.2 to about 0.5 mm in diameter. Using this device, the cells were passed through a significantly smaller diameter passageway than has previously been used, with a result that the cell velocity increased as the diameter of the passageway narrowed. The narrowing of the exit tip of the pipette generated the greater fluid velocities for introduction of the genetic material into the living cells. Further, the highly polished end was not observed to have caused loss of cell viability. A low calcium concentration in the solution protected the cells from calcium-induced cell death, while prolonging the opening of membrane holes or pores.

Examples herein were obtained with neurons and non-neuronal cells using magnesium/calcium solutions generally having 1.5 mM magnesium/200 nM calcium. Neurons are known to lose viability following prolonged calcium influx associated with activation of calcium-activated proteases. No signs of cell death were observed in the neuronal cultures following use of the methods and these solutions, monitored for a period of 48 hours.

Example 3: Introducing genetic material into living cells

Ciliary ganglia from chick embryos were used in the example herein. The ganglia were dissociated from each other using a larger bore glass pipette, with positive and negative pressure being manually applied using a rubber pipette bulb.

Cells were placed in a small volume (50 µl to 100 µl), and a solution having a high concentration of plasmid cDNA (70 µg/ml) containing a gene that encodes green fluorescent protein (GFP) fusion proteins of interest was added to the cells .

A very fine bore polished pipette was then used to pass the cells through the tip at high velocity. The fluid was passaged sequentially back and forth through the small bore tip

about 5 times to about 10 times, into a vessel or receptacle, in this example a 1500 µl Eppendorf microcentrifuge tube. The small volume of cells and plasmid cDNA was almost entirely passaged, which maximized exposing the cells to the DNA during the high velocity transitions. The passaging process was performed while monitoring volumes and minimizing production of bubbles, as extracellular oxygen is toxic to cells. As the cells were passed through the tip of the pipette, pressure was gradually increased, which prevented bubble formation and improved cell survival among cells in the population treated by the technique. The method in Examples herein maintained a constant fluid pressure, so as to avoid sudden, instantaneous changes in fluid pressure which damage cells.

Further, the method for initial dissociation and high velocity passaging of the cells was performed in a solution that was substantially free of or had a low concentration of calcium, and that included a high concentration of magnesium (from 1.5mM to 5.0mM). This solution was designed to protect the cells from calcium influx, calcium challenges, and resultant calcium-mediated cell death through activation of calcium-dependent proteases. Such activation of calcium-dependent proteases is specific to the type of cell treated, and therefore higher or lower concentrations of calcium or magnesium were used in Examples herein according to the type of cell.

Following passaging with nucleic acid, the cells were collected by centrifugation at 3000 rpm in a microcentrifuge and the supernatant was removed from the cell pellet, and was replaced with fresh culture medium. The cell pellet was then gently re-suspended in the medium, and the cell suspension was cultured on plates. GFP gene and a GFP fusion protein were expressed and fluorescence was observed within three to six hours in the cell bodies of each of the neuronal and the nonneuronal cells found in the ganglia population. Fig. 1 and Fig. 2.

A previously shown method using a vector constructed from Sindbis virus was performed as a control. Recipient cells did not show visible minimal expression until about 12 hours after contacting the cells. The methods provided in Examples herein achieved visible GFP expression, and expression was clearly observed with high resolution low-light CCD fluorescence microscopy within a fraction of time than the method using the vector constructed from Sindbis virus, i.e., lag time was reduced by about 50% to about 75%.

Example 4: Expression of myosin Va

A gene encoding a GFP-myosin V fusion protein was introduced into chick ciliary ganglion neurons. Movement of GFP-myosin Va-positive membrane compartments within

axons and cell bodies was observed and the localization of GFP-myosin Va was consistent with the localization of myosin V in control untreated cells.

Myosin Va is a large multi-domain protein (190 kD), therefore rapid expression from a relatively long plasmid required of the genes encoding such a large protein, was surprising. Expression of GFP-myosin Va was observed initially within three hours, and by six hours punctate or localized compartments of fluorescence were observed. These data show that expression of the myosin was associated with directed motion that is typical of membrane compartments, the motion conveyed by the motor activity of myosin Va or associated motor proteins such as kinesin, which bring myosin Va-linked membrane compartments to the periphery along microtubules. Distribution and amount of expression of the GFP-myosin Va construct and the GFP construct each increased in abundance slowly during 24 hours following introduction of the construct.

Example 5: Kinetics and localization of expression of genetic material introduced into living cells

Expression of GFP was observed to increase slowly during 24 hours following contacting the cells with constructs. At 24 hours, GPF protein was observed to be diffuse throughout the cell, and in intensely-fluorescent membrane compartments within the cytoplasm of the cell. Fig. 1.

Table 1 below shows the frequency (number of cells randomly sampled, and percent) of GFP protein control expression (GFP) or GFP-myosin Va construct (Myosin Va) expression in neuronal and nonneuronal cells, observed as a function of time after transfection using the method herein, including plating the cells in culture. It was observed that nonneuronal cells required a greater time period for appreciable expression, and that neurons were observed to show consistently high frequencies of expression in either a diffuse form early after culturing, or in the form of punctate or compartmentalized staining later after culturing. Without being limited by any particular theory or mechanism of action, it is envisioned that the observed staining is related to a relatively higher metabolic activity of growing neurons in culture.

Expression of GFP protein in nonneuronal and neuronal cells was determined to achieve a maximum at 48 hours after the time of loading/introduction (See Fig. 2). GFP protein expression was observed to be abundant in compartments in the cytoplasm, and was mostly absent from the nucleus. Fig. 2.

Strong evidence of localization of myosin Va was observed both in the cell body and the neuronal processes. Localization was observed particularly in branch points and tips of growing axonal processes. Fig. 3 Panel A and Panel B.

Data in Examples herein demonstrate successful transfection of neuronal cells with genes encoding different myosin Va constructs, with substantial expression observed in the cells. Transfection was observed to result in specific changes in nerve cell projections at the growing tips. Specifically, changes were observed in neuronal filopodia, the finger-like projections of growing tips (Fig. 4).

Table 1. Frequency in cells of gene expression observed as a function of time

Neurons						
Time (h) after culturing	GFP stain	GFP total	GFP %	Myosin Va stain	Myosin total	Myosin Va %
3	5	5	100	17	17	100
4	18	18	100	15	15	100
5				23	26	88.5
7	44	45	97.8	30	30	100
21				22	22	100
Nonneuronal cells						
Time (h) after culturing	GFP stain	GFP total	GFP %	Myosin Va stain	Myosin total	Myosin Va %
3	0	4	0	5	18	27.8
4	3	6	50	0	2	0
5				11	20	55
7	26	32	81.2	21	37	56.8
21				33	35	94.3

Example 6: Expression of genetic material in mouse sperm cells

As a test of this technology, the methods provided in Examples herein were applied to a cell system, the mature sperm cell, in which introduction and expression of genetic material has not previously been reported.

Sperm cells are notoriously difficult to permeate, and also are characterized as having no nuclear translation and very little RNA. Protein synthesis in sperm cells is confined to mitochondria within the small amount of mature sperm cytoplasm. Thus, sperm cells have largely been considered inaccessible to methods of recombinant DNA technology.

The methods and devices in Examples herein were used to introduce a gene encoding pCherry, a variant of GFP protein, the variant having fluorescence in the red part of the

visible spectrum (Shaner et al., Nat Biotechnol 22(12): 1567-1572, 2004). The pCherry gene is operatively linked to and controlled by a CMV promoter. Following transfection the living mouse sperm cells were permitted to recover overnight at 37 °C. Within 24 hours pCherry fluorescence was observed in a subpopulation of treated sperm cells (Fig. 5). These data show for the first time successful expression of a recombinant gene, encoding pCherry protein, in the sperm cells treated by the methods herein.

Localization of fluorescence of GFP-positive sperm cells was compared with that of a set of control sperm cells treated with a membrane permeable fluorescent dye, "Lavacell", which permeates cells and stains internal membranes. Fluorescence of Lavacell stained sperm cells was observed throughout the cell including the cell head, midpiece and tail (See Fig. 5).

In contrast, transfected pCherry expressing sperm cells (not treated with Lavacell) showed essentially no fluorescence in the head portion of the cells. These cells were observed to have intense fluorescence in the midpiece where mitochondria are abundant, with minimal fluorescence in the flagellum or tail segment (Fig. 5). pCherry expression was observed to be present in 75% of the sperm cells at sites of localization, consistent with localization of RNA in sperm cells.

Example 7: Introducing genetic material into cells using pressurized solutions

Genetic material is introduced into a cell in a tissue or in a monolayer in culture by ejecting a composition including a genetic material onto the cells in the tissue or monolayer. The genetic material is introduced into the cell/monolayer of cells using fluid pulsed at high velocity which creates conditions similar to that of the methods described herein, such that the increase in fluid speed corresponds to a simultaneous decrease in pressure at the cell membrane. The pulsed fluid momentarily stretches the cell membrane (Bernoulli's principle) and a pore or a plurality of pores is formed that acts as a point of entry for the genetic material.

The fluid containing the genetic material is pulsed at a specific pressure, frequency, or force to form a pressure wave. Alternatively, the fluid is pulsed across the surface of, or directly at the face of, the tissue or monolayer.

It is envisioned that genetic engineering in situ of tissues needing a therapeutic gene can be achieved by this method, for example, a gene encoding a normal allele of a defective inherited gene, for example, into a hematopoietic tissue such as bone marrow or liver, can be delivered by this method.

What is claimed is:

1. A pressurized fluid flow apparatus comprising: a pipe portion having an inner diameter and an outer diameter and a distal end and a proximal end, wherein the distal end is open to the atmosphere, wherein the inner diameter of the pipe portion is about 1.0 mm to about 5.0 mm; the apparatus further comprising an exit tip adjacent to and contiguous with the distal end of the pipe portion, wherein the exit tip has an inner diameter and an outer diameter, the inner diameter of the exit tip being about 0.1 mm to about 0.5 mm; and a suction device connected to the proximal end of the pipe portion for generating positive pressure and negative pressure.

2. The apparatus according to claim 1, further comprising a receptacle adjacent to the exit tip for receiving and retaining fluid passaged through the pipe portion and exit tip.

3. The apparatus according to claim 1, wherein the pipe portion and the exit tip comprise at least one material selected from the group consisting of: borosilicate glass, aluminosilicate glass, and the like.

4. The apparatus according to claim 1, wherein the distal end of the exit tip is polished by heat treatment to create a non-lacerative surface.

5. The apparatus according to claim 4, wherein the heat treatment is flame-polishing.

6. The apparatus according to any of claims 1 to 5, wherein the suction device generates a flow velocity along the length of the pipe portion, the flow velocity selected from at least one of about: 5 cm/s, 15 cm/s, and 20 cm/s.

7. The apparatus according to any of claims 1 to 5, wherein the suction device generates a mass flow rate at the exit tip, the rate selected from at least one of a group consisting of about: 0.03 ml/min, 0.06 ml/min, 0.3 ml/min, 0.5 ml/min, 1.0 ml/min, and 2.0 ml/min.

8. The apparatus according to any of claims 1 to 5, wherein the suction device generates a mass flow rate along the length of the pipe portion, the rate selected from at least one of a

group consisting of about: 1.0 ml/min, 5 ml/min, 10 ml/min, 50 ml/min, 100 ml/min, and 150 ml/min.

9. The apparatus according to any of claims 1 to 5, wherein the suction device is manually controlled.

10. The apparatus according to any of claims 1 to 5, wherein the suction device is automated.

11. The apparatus according to any of claims 1 to 5, wherein the exit opening further comprises a constriction in a continuous flow system controlled by at least one valve.

12. The apparatus according to claim 11, wherein the at least one valve is a plurality of valves positioned laterally distal or laterally proximal to the exit.

13. A method for introducing genetic material into a cell, the method comprising:

contacting in a receptacle at least one cell with a composition comprising an effective amount of the genetic material to obtain a resulting mixture; and,

inserting the apparatus of claim 1 into the receptacle and passaging the mixture of cells and genetic material through the apparatus at least once, wherein passaging the mixture having the cells and the genetic material through the apparatus introduces the genetic material into the cell.

14. The method according to claim 13, wherein the cell is a prokaryotic cell.

15. The method according to claim 13, wherein the cell is a eukaryotic cell.

16. The method according to claim 13, wherein introducing the genetic material is localizing the genetic material to the nucleus of the cell.

17. The method according to claim 13, wherein introducing the genetic material is localizing the genetic material into the cytoplasm of the cell.

18. The method according to either of claims 16 and 17, wherein localizing further comprises visualizing the genetic material in the cell with a detectable marker.

19. The method according to claim 18, wherein the detectable marker is selected from the group consisting of: detectable, fluorescent, colorimetric, enzymatic, radioactive, and the like.

20. The method according to claim 13, wherein passaging the mixture further comprises dispensing at least once into the receptacle.

21. The method according to claim 13, wherein passaging the mixture further comprises dispensing into a plurality of receptacles.

22. The method according to claim 13, wherein the cell is found in a population of a plurality of cells.

23. The method according to claims 22, wherein the plurality of cells are living cells, and the viability is not substantially reduced.

24. The method according to claim 13, wherein the receptacle is a centrifuge tube.

25. The method according to claim 13, wherein the composition comprises a calcium concentration less than about 200 nM.

26. The method according to claim 13, wherein the composition comprises a magnesium concentration of at least about 1.5 mM.

27. The method according to claim 13, further comprising, after passaging, centrifuging the mixture to obtain a cell pellet and supernatant.

28. The method according to claim 27, further comprising removing the supernatant, adding cell culture medium to the receptacle, and re-suspending the cell pellet in the medium.

29. The method according to claim 28, further comprising culturing the cells.

30. The method according to claim 22, wherein the cells are living postmitotic cells.

31. The method according to claim 30, wherein the postmitotic cells are neurons or sperm cells.

32. The method according to claim 31, wherein the neurons are ciliary ganglion neurons or dorsal root ganglion neurons.

33. The method according to claim 22, wherein the cells are living premitotic cells.

34. The method according to claim 33, wherein the premitotic cells are at least one cell type selected from the group consisting of epithelial, hematopoietic, liver cells, and spleen cells.

35. The method according to claim 22, wherein the cells are physiologically inactive.

36. The method according to claim 35, wherein the physiologically inactive cells are selected from the group of: inhibited, UV-inactivated, enucleated, anucleate, and heat-killed.

37. The method according to claim 13, wherein the genetic material is DNA or RNA

38. The method according to claim 37, wherein the DNA is cDNA.

39. The method according to claim 37, wherein the RNA is at least one selected from the group consisting of mRNA, tRNA, rRNA, siRNA, RNAi, miRNA, and dsRNA.

40. The method according to claim 13, wherein the composition further comprises at least one transfection agent.

41. The method according to claim 40, wherein the at least one transfection agent is selected from the group consisting of: a nanoparticle, a liposome, a viral vector, a bacteriophage, and a detergent.

42. A kit for introducing genetic material into a nucleus of a living cell comprising: the apparatus of claim 1.

43. The kit according to claim 42, further comprising a receptacle.

44. The kit according to either of claims 42 or 43, further comprising instructions for use.

45. The kit according to any of claims 41 to 44, further comprising a transfection agent.

46. A method for introducing genetic material into at least one cell in a tissue or a monolayer in culture, the method comprising:

inserting the apparatus of claim 1 into a receptacle, wherein the receptacle contains a composition comprising an effective amount of the genetic material; and,

contacting the at least one cell in the tissue or the monolayer of cells in culture, wherein contacting comprises ejecting the composition under pressure onto the cell or cells, whereby the genetic material is introduced into the cell in the tissue or the cells of the monolayer.

47. The method according to claim 46, wherein ejecting composition under pressure comprises generating a pressure wave having a particular frequency.

48. The method according to claim 46, further comprising, following contacting, observing the genetic material entering the at least one cell without disrupting cell membranes or tissue.

49. The method according to claim 46, wherein the cell is a prokaryotic cell.

50. The method according to claim 46, wherein the cell is a eukaryotic cell.

51. The method according to claim 46, wherein ejecting is localizing the genetic material to the nucleus of the cell.

52. The method according to claim 46, wherein ejecting is localizing the genetic material to the cytoplasm of the cell.

53. The method according to claims 51 or 52, wherein localizing comprises visualizing the genetic material in the cell with a detectable marker.

54. The method according to claim 53, wherein the detectable marker is selected from the group consisting of: detectable, fluorescent, colorimetric, enzymatic, radioactive, and the like.

55. The method according to claim 46, wherein the cell is within a population of a plurality of cells.

56. The method according to claims 55, wherein the plurality of cells is a plurality of living cells, and the viability of the living cells is not substantially reduced.

57. The method according to claim 46, wherein the genetic material is DNA or RNA

58. The method according to claim 57, wherein the DNA is cDNA.

59. The method according to claim 57, wherein the RNA is at least one selected from the group consisting of mRNA, tRNA, rRNA, siRNA, RNAi, miRNA, and dsRNA.

60. The method according to claim 46, wherein the tissue is a mammalian tissue.

61. The method according to claim 60, wherein the mammalian tissue is a human tissue.

62. The method according to claim 61, wherein the human tissue is at least one selected from the group consisting of: skin, kidney, pancreas, liver, lung, heart, brain, spinal cord, bone marrow, and eye.

63. The method according to claim 45, wherein the monolayer of cells in culture comprises stem cells.

64. The method according to claim 63, wherein the stem cells are selected from at least one of hematopoietic, hemangioblast, mesenchymal, hepatocyte, pancreatic, pulmonary, neural, fetal, and embryonic stem cells.

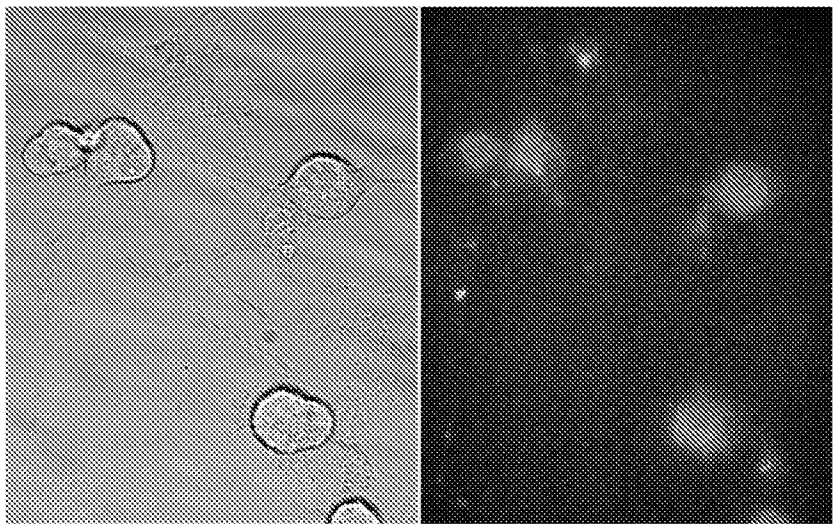


Fig. 1

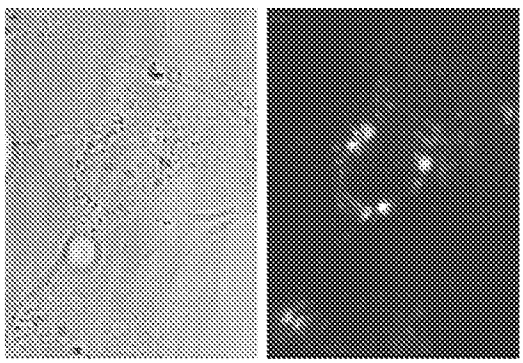


Fig. 2A

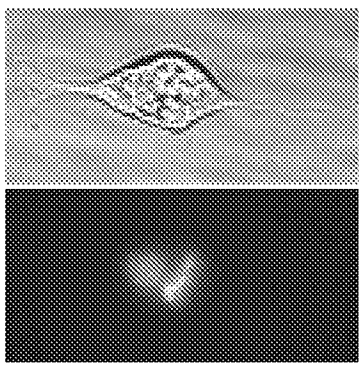


Fig. 2B

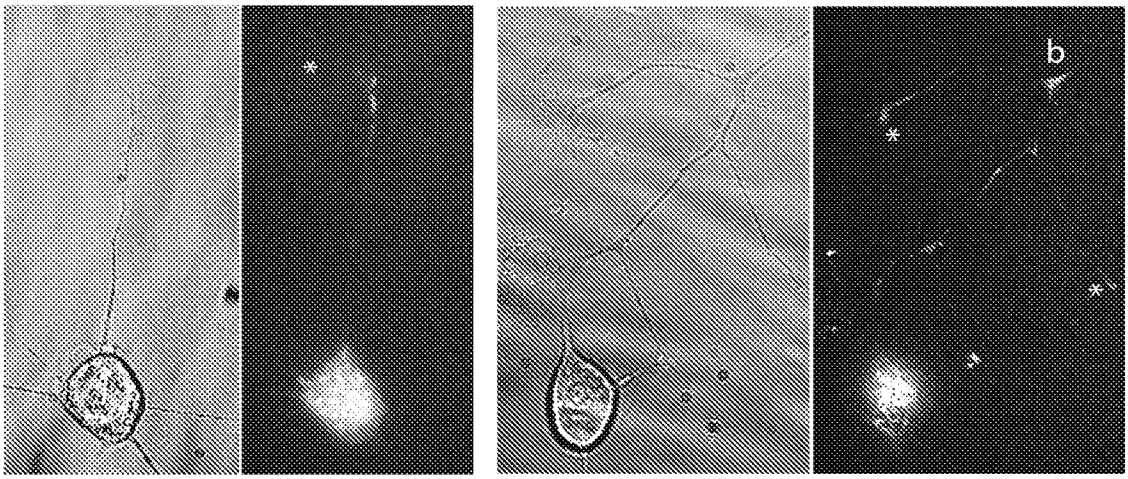


Fig. 3

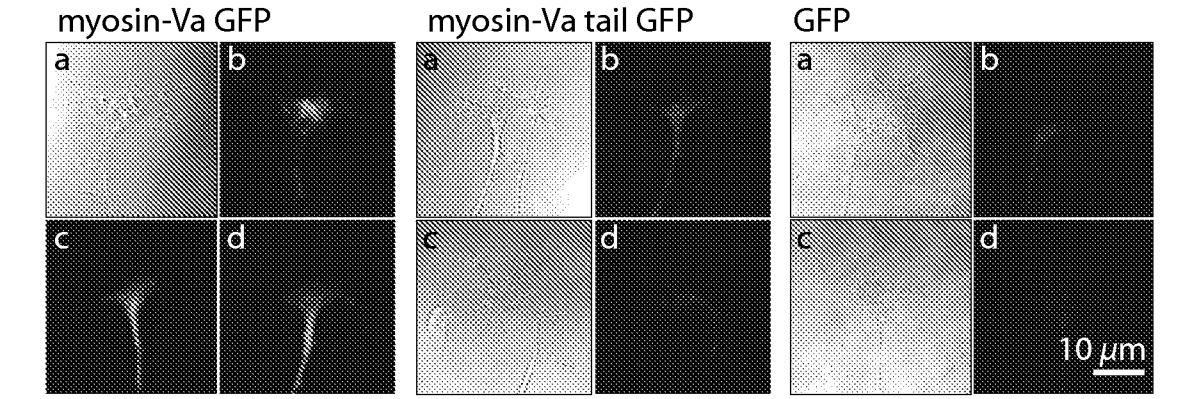

myosin-Va GFP
myosin-Va tail GFP
GFP
a
b
c
d
10 μm

Fig. 4A

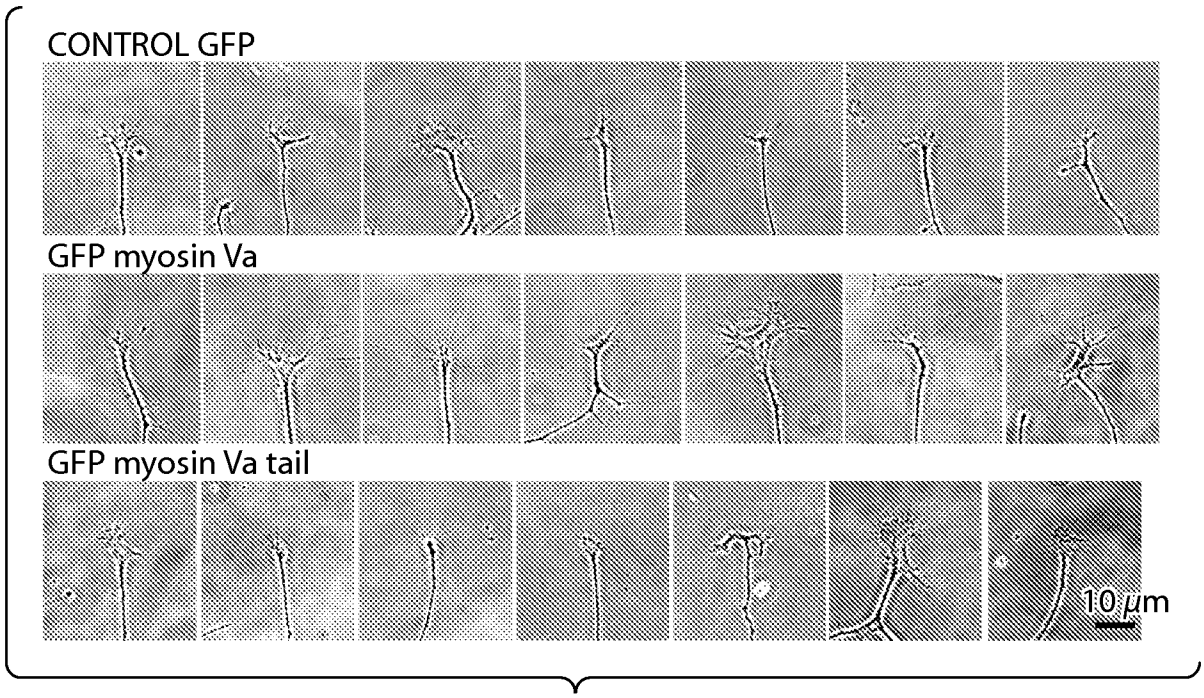
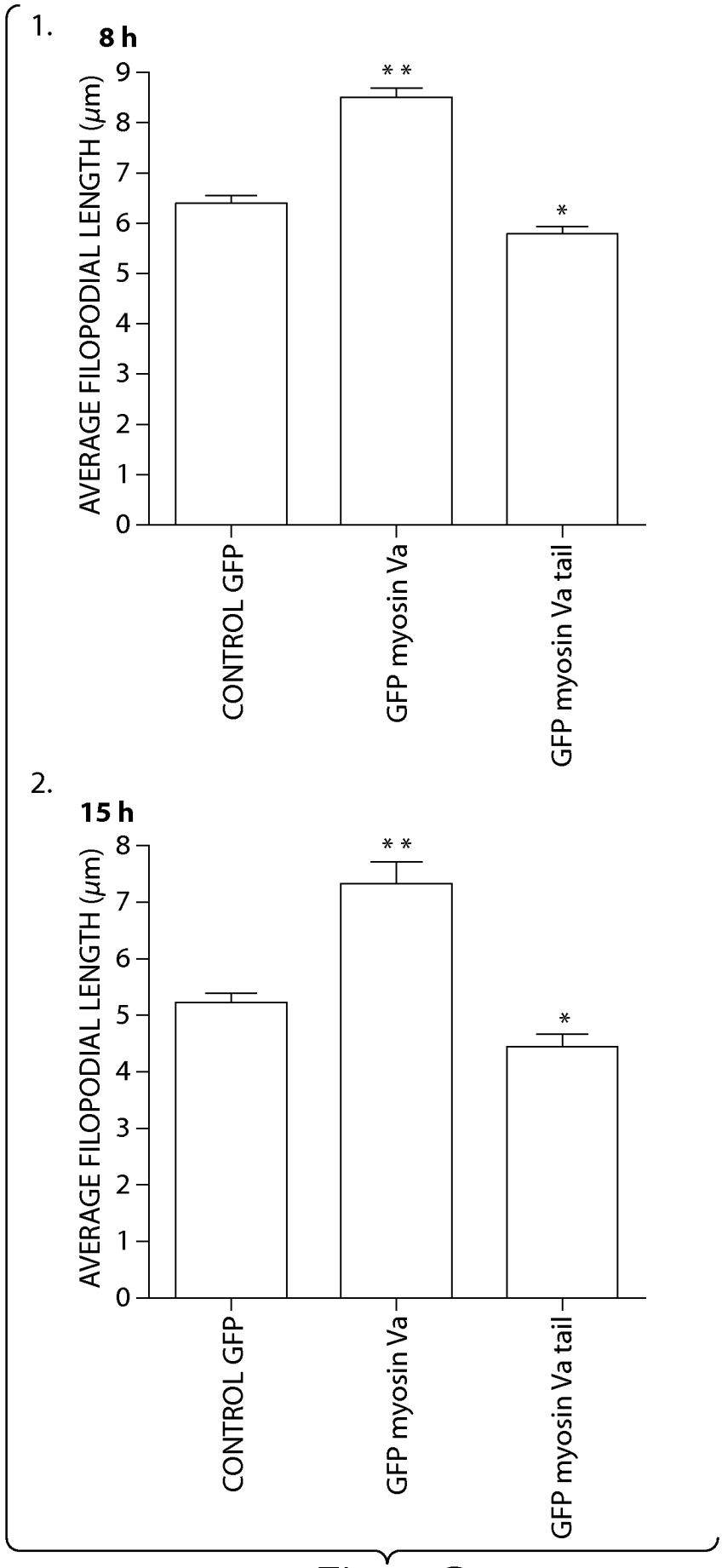

CONTROL GFP
GFP myosin Va
GFP myosin Va tail
10 μm

Fig. 4B


1.
8 h
AVERAGE FILOPODIAL LENGTH (μm)
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CONTROL GFP
GFP myosin Va
GFP myosin Va tail
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15 h
AVERAGE FILOPODIAL LENGTH (μm)
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GFP myosin Va tail

Fig. 4C

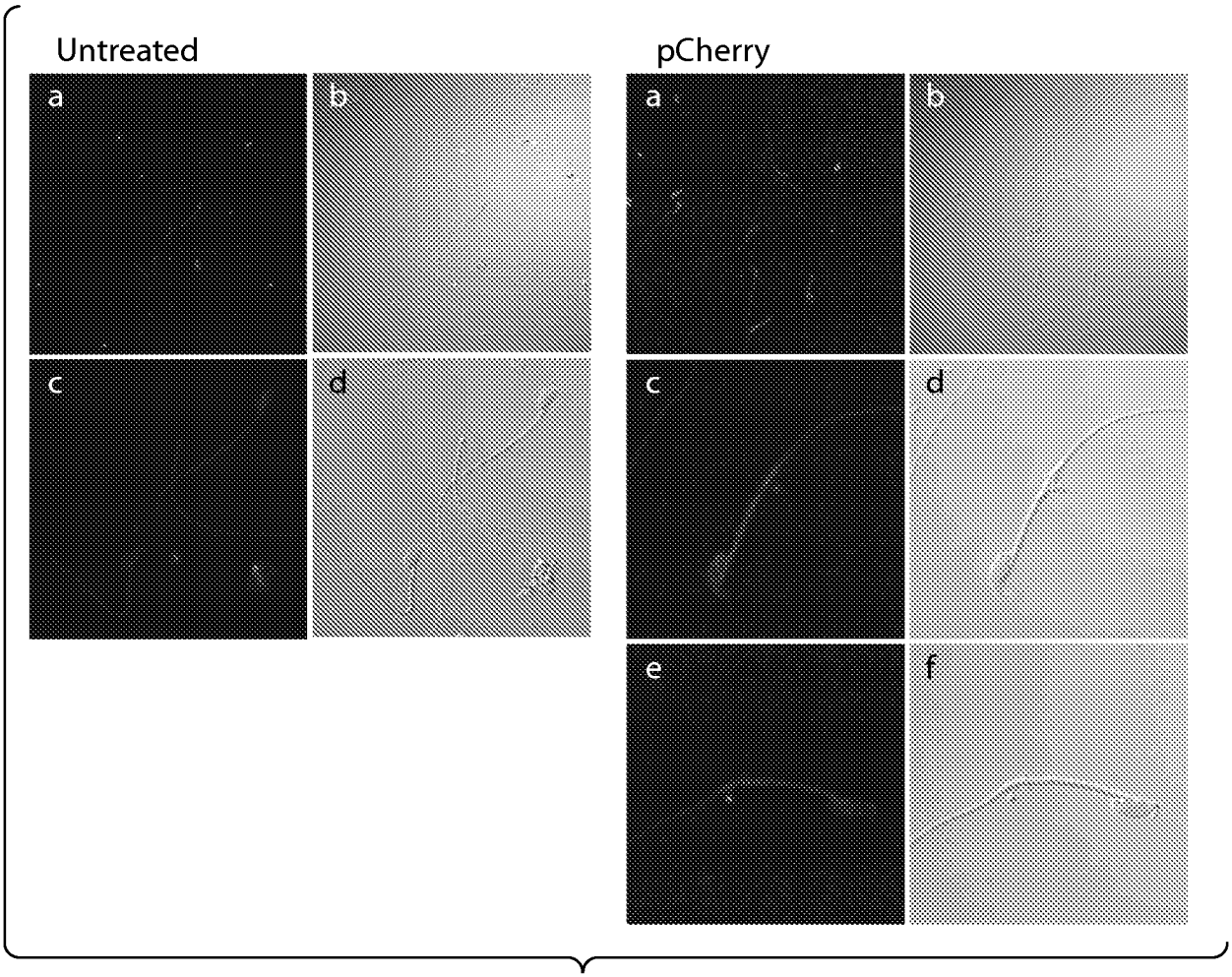

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/27104

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61M 5/00, C12M 3/00 (2010.01)
USPC - 604/264, 435/285.1, 435/286.5, 435/288.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)- A61M 5/00, C12M 3/00 (2010.01)
USPC-604/264, 435/285.1, 435/286.5, 435/288.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPS- 604/19

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar
transfection, auotomatic, manual, pipette, pipettor, eukaryot$, prokaryot$, pressure wave, high frequency, pressurized fluid flow, nucleic acid, RNA, DNA, valve

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 6,982,063 B2 (HAMEL et al.) 03 January 2006 (03.01.2006) col 4 ln 45-55; col 5 ln 5-10; col 15 ln 1-67; col 16 ln 1-55; col 17 ln 1-30; col 19 ln 1-15; Figs. 16, and 20-23	1-12, 42-44 --- 13-41, 46-64
Y	TSCHOEP et al., Shock waves: a novel method for cytoplasmic delivery of antisense oligonucleotides. J Mol Med, June 2001, vol 79, no 5-6, pp306-313. (pg 307 col 1, 2; pg 308 col 2 para 1-3)	13-41, 46-64

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 April 2010 (08.04.2010)	26 APR 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/27104

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

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

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

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

3. ☒ Claims Nos.: 45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.