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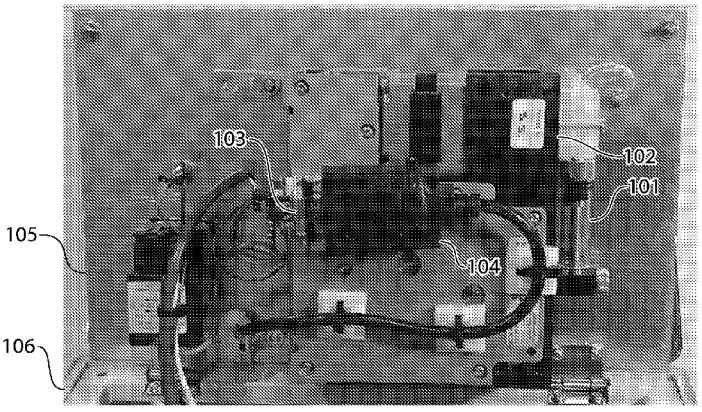

Fig. 1A

(57) **Abstract**: Systems, tip assemblies, methods and kits are provided for introducing material into cells. The tip assemblies include an attachment portion, a channel portion, and a constriction that function to reduce fluid pressure as a fluid passes through the constriction portion from the channel portion, whereby the tip assemblies form pores in the membranes of cells and introduce material into the cells. The material includes for example one selected from the group of: an inorganic compound, a drug, a genetic material, a protein, a carbohydrate, a synthetic polymer, and a pharmaceutical composition.

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SYSTEMS, TIP ASSEMBLIES, METHODS AND KITS FOR INTRODUCING MATERIAL INTO CELLS

Related applications

This application claims the benefit of U.S. provisional application serial number 61/438,824 filed February 2, 2011, entitled "Methods, tip assemblies and kits for introducing material into cells", inventor Thomas Diefenbach, and U.S. continuation-in-part application serial number 13/231,592 filed September 13, 2011, which shares the same title and inventor, and which are hereby incorporated herein by reference in their entireties.

Technical field

Systems, tip assemblies, methods and kits are provided for introducing material into cells. The material introduced into cells includes an inorganic compound, a drug, a genetic material, or a pharmaceutical composition.

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, baculo virus 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 embodiment of the invention provides a system for introducing a composition in a fluid into cells, the system including; a flow device that generates at least one of a positive pressure and a negative pressure for impelling the fluid; and, a tip assembly for passaging a mixture of the cells and the fluid including: an attachment portion that proximally connects to the flow device, a channel portion contiguous to and distal to the attachment portion and the flow device, a constriction portion contiguous with the channel portion, such that a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, such that a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, such that increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, such that the system introduces the composition into the cells through the membrane. For example, an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion
enhances formation of membrane pores in the cells, and the system introduces the composition into the cells through the membrane pores.

The system, in another embodiment further includes a receptacle adjacent to the opening for receiving the fluid passaged through the tip assembly.

In related embodiments of the system, the tip assembly includes at least one selected from the group consisting of: a glass, a metal, a plastic, a polymer, a nano-based composition, a composite material comprising at least two different types of substances, and the like; optionally, the flow device includes at least one selected from the group consisting of: a syringe, a plunger, a bulb, a diaphragm, and a compressor; optionally, operation of the flow device comprises control of at least one selected from the group of: positive fluid pressure; negative fluid pressure; flow velocity; flow acceleration; mass flow rate; initial velocity ramp; starting velocity; maximum velocity; starting position; cutoff velocity period of time the fluid is under pressure generated by the flow device; temperature, and period of time the fluid is held in the channel portion, constriction portion, or both; optionally, the flow device generates a flow velocity along the length of the tip assembly, the flow velocity selected from about: about 0.1 cm/s to about 1 centimeter per second (cm/s), about 1 cm/s to about 5cm/s, about 5cm/s to about 15 cm/s, and about 15 cm/s to about 20 cm/s; such that the flow device generates a mass flow rate selected from a group consisting of about: 0.01 milliliter per minute (ml/min), 1 ml/min, 10

ml/min, 25 ml/min, 50 ml/min, 100 ml/min, and 150 ml/min; and wherein the flow device generates an acceleration in the tip assembly of about 0.6 µl/s/s, about 1 µl/s/s, about 6 µl/s/s, about 10 µl/s/s, about 12 µl/s/s, about 24 µl/s/s, about 36 µl/s/s, about 48 µl/s/s, and about 60 µl/s/s.

According to one embodiment of the system, the flow device is controlled or operated manually. According to another embodiment, the flow device is at least one selected from the group of: automated, electromechanical, and programmable.

In various related embodiments, the system further includes at least one selected from the group of: a power source; a connector for interacting with a user interface, a computer, a hand-held device, a transmitter, or a display; a conduit for connecting the flow device to the tip assembly; a sensor, a valve; and a housing.

Another embodiment of the invention is a tip assembly for introducing a composition in a fluid into cells having: an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid; a channel portion contiguous to and distal to the attachment portion and the flow device; a constriction portion contiguous with the channel portion, such that a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, such that a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, such that increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, such that the tip assembly introduces the composition into the cells through the membrane. For example, an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion enhances formation of membrane pores in the cells, and the tip assembly introduces the composition into the cells through the membrane pores.

In related embodiments, the tip assembly is selected from at least one of: disposable, modular, transparent, and translucent; optionally, the tip assembly is reusable; optionally, at least one of the attachment portion, the channel portion, and the constriction portion comprises a substance selected from the group of: a glass, a metal, a plastic, a polymer, a nano-based composition, a composite material comprising at least two different types of substances, and the like; optionally, an inner diameter or outer diameter of the attachment portion of the tip assembly fits the flow device in a male to female arrangement; optionally, the channel portion cross sectional area or the constriction portion cross sectional area is bounded by a continuous circle, an ellipse, a rectangle or a regular polygon such as a square, and the circle polygon or

other cross sectional area shapes are continuous or discontinuous, and such that the distal end of the constriction portion comprises an inner diameter that is less than or substantially equal to an inner diameter of the opening; optionally the tip assembly contains a volume which is: about 2 microliters (μl), about 20 μl, about 50 μl, about 200 μl, about 400 μl, about 500 μl, about 1 milliliter (ml), about 5 ml, and about 10 ml, and wherein the channel portion inner diameter is about 1.0 millimeter (mm) to about 10.0 mm, and the diameter of the constriction inner diameter is about 0.05 mm to about 2.0 mm, wherein the constriction inner diameter is smaller than the channel portion inner diameter.

According to related embodiments of the tip assembly, the channel portion curvature and constriction inner curvature characterize a fluid path for the fluid flowing through the tip assembly, such that the channel portion curvature and constriction inner curvature necessary for introducing the composition into the cells includes a formula represented in two dimensions by

$$f(x) = p1x^7 + p2x^6 + p3x^5 + p4x^4 + p5x^3 + p6x^2 + p7x + p8$$

such that x is a radial distance from a center axis on the fluid path to an inner surface of the tip and coefficients with 95% confidence bounds in parentheses comprise: p1 is $-2.611e^{-16}$ ($-6.043e^{-16}$, $8.206e^{-17}$), p2 is 3.954^{e-13} (-3.195^{e-13}, 1.11^{e-12}), p3 is -1.845^{e-10} (-7.821^{e-10}, 4.131^{e-10}), p4 is 1.662^{e-08} (-2.394^{e-07}, 2.726^{e-07}), p5 is 7.537^{e-06} (-5.186^{e-05}, 6.694^{e-05}), p6 is -0.002137 (-0.009375, 0.005101), p7 is -0.003185 (-0.4114, 0.4051), and p8 = 268.6 (261, 276.3), or

$$f(x) = p1x^8 + p2x^7 + p3x^6 + p4x^5 + p5x^4 + p6x^3 + p7x^2 + p8x + p9$$

such that x is a radial distance from a center axis on the fluid path to an inner surface of the tip and coefficients with 95% confidence bounds in parentheses comprise: p1 is 2.285 (1.388, 3.182), p2 is 3.465 (1.782, 5.149), p3 is -15.68 (-20.04, -11.32); p4 is -20.38 (-27.24, -13.52); p5 is 44.27 (36.5, 52.04); p6 is 53.96 (45.69, 62.23); p7 is -58.72 (-64.02, -53.42), p8 is -123.5 (-126.4, -120.6), and p9 is 186.5 (185.6, 187.4).

The tip assembly, in another embodiment of the invention, further includes a shoulder extending laterally from of an outward surface of the attachment portion, for example, a lower ejector section of the flow device removes the tip assembly from the flow device.

In another embodiment of the invention, the constriction portion of the tip assembly that connects to the flow device includes a continuous channel leading to a second channel through which fluid then flows toward an exit portion of the system which optionally further includes any of a storage reservoir, a cell processing unit and the like. In this embodiment the fluid flows

from an entry channel, through the constriction, then through an exit channel in a continuous flow-through system.

Another embodiment of the invention is a method for introducing a composition in a fluid into cells, the method including: contacting the cells in a reservoir with the fluid comprising the composition; inserting a tip assembly into the reservoir, wherein the tip assembly includes: an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid; a channel portion contiguous to and distal to the attachment portion and the flow device; a constriction portion contiguous with the channel portion, such that a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, such that a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, such that increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells; and passaging a mixture of the cells and the fluid with the composition at least once through the tip assembly using a flow device, such that passaging the mixture introduces the composition into the cells through the membrane. For example, an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion enhances formation of membrane pores in the cells; and passaging a mixture of the cells and the fluid with the composition at least once through the tip assembly using a flow device, and passaging the mixture introduces the composition through the pores into the cells.

In related embodiments of the method, passaging the mixture further includes redirecting the mixture to the reservoir; optionally, passaging the mixture further includes dispensing the mixture into a receptacle; optionally, after passaging the mixture through the constriction of a continuous channel, the mixture then proceeds continuously through an exit channel; optionally, the cells are a population of a plurality of living cells, optionally eukaryotic cells, and the method further includes observing maintenance of cell viability, the cell viability is not substantially reduced and includes at least about: 1%, 10%, 30%, 50%, 75%, 90%, or 95% of control cells not passaged through the tip assembly; optionally, the fluid comprises a Ca^{-2} concentration less than about 200 nanomolar (nM), about 150 nM, about 100 nM, or about 75 nM, and wherein the fluid comprises a Mg^{+2} concentration less than about 200 nM, about 150 nM, about 100 nM, or about 75 nM; optionally such that the composition comprises a DNA or an RNA, such that the DNA is selected from the group of: native DNA, synthetic DNA, cDNA, dsDNA, ssDNA and the RNA is selected from the group of: mRNA, tRNA, rRNA, siRNA,

RNAi, miRNA, and dsRNA, or a portion thereof, and the method further involves assaying transfection of the cells.

According to other related embodiments, the method further includes after passaging, observing localization of the composition to at least one subcellular compartment selected from: a nucleus; a mitochondrion; a Golgi body; an endoplasmic reticulum; a chloroplast; a chromoplast; an endosome, a vesicle, a vacuole, a lysosome, an axon; a cytoplasmic membrane; a nuclear membrane; a cytoskeleton and a cytoplasm, such that observing the localization further includes at least one of: visualizing the composition with a detectable marker selected from the group consisting of: detectable, fluorescent, colorimetric, enzymatic, radioactive; and quantifying directly the product of the composition that entered the cell from the group consisting of: mRNA, DNA, RNA, and protein; optionally, such that the cells comprise a cell type selected from the group consisting of: epithelial cells, hematopoietic cells, stem cells, spleen cells, kidney cells, pancreas cells, liver cells, neuron cells, glial cells, human umbilical vein endothelial cells (HUVEC), smooth or striated muscle cells, sperm cells, heart cells, lung cells, ocular cells, bone marrow cells, fetal cord blood cells, progenitor cells, peripheral blood mononuclear cells, leukocyte cells, lymphocyte cells, living postmitotic cells, physiologically inactive cells, inhibited cells, UV-inactivated cells, enucleated cells, anucleate cells, heat-killed cells, non-reproducing cells, and synthetic cells having an artificial membrane or the like.

The method, in a related embodiment, further includes after passaging, centrifuging the mixture to obtain a cell pellet and a supernatant, removing the supernatant, adding culture medium to the reservoir, re-suspending the cell pellet in the medium, and culturing the cells.

In other related embodiments, the method further includes applying to the mixture at least one selected from the group of: an electric field, a light or radiation characterized by a wavelength, and a sound pulse.

Another embodiment of the invention is a kit for introducing a composition in a fluid into cells including: a tip assembly for passaging a mixture of the cells and the fluid comprising: an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid; a channel portion contiguous to and distal to the attachment portion and the flow device; a constriction portion contiguous with the channel portion, such that a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, such that a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, such that increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, such that the tip

assembly introduces the composition into the cells through the membrane; and a container. For example, an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion enhances formation of membrane pores in the cells, and the tip assembly introduces the composition into the cells through the membrane pores.

In a related embodiment, the kit has instructions for use including: contacting the cells in a reservoir with the fluid comprising the composition; inserting the tip assembly into the reservoir; contacting the attachment portion of the tip assembly to a flow device that generates at least one of a positive pressure and a negative pressure; and passaging a mixture of the fluid and the composition at least once through the tip assembly using the flow device, and passaging the mixture affects membrane permeability of the cells and introduces the composition into the cells. For example, passaging the mixture of the fluid and the composition at least once through the tip assembly using the flow device forms membrane pores in the cells and introduces the composition into the cells. In another related embodiment, the kit further includes a reservoir; optionally further includes a transfection agent, a buffer, or a medium, cell line or strain.

Another embodiment of the invention is a flow-through assembly for introducing a composition in a fluid into cells including: an entry channel portion open to the atmosphere that connects to and is proximal to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid into the entry channel portion; a constriction portion contiguous to and distal to the entry channel portion and the flow device; an exit channel portion contiguous with the constriction portion and distal to the flow device and the constriction portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than inner diameters and cross sectional areas respectively of the entry channel portion and the exit channel portion respectively, wherein a distal end of the exit channel portion has an opening for ejecting or drawing the fluid, wherein the fluid flows through the constriction portion in either direction, and wherein an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the entry or the exit channel portion affects membrane permeability of the cells, such that the flow-through assembly introduces the composition into the cells through the membrane.

Brief description of the drawings

Fig. 1 is a set of photographs of an exemplary transfection system.

Fig. 1 panel A is a photograph of a computer programmable syringe pump **101**, **102** connected to an external computer by means of a USB converter **103** and an RS232 interface **104** configured in the inner side of the metal lid **105** of a jump box **106**. The RS232 interface **104** to USB converter **103** connection is linked by a cable to an external computer for control using the data analysis software MATLAB (Mathworks, Natick, MA).

Fig. 1 panel B is an enlarged view of the programmable syringe **101** and the pump mechanism **102** positioned on the jump box **106** right. The exemplary syringe **101** is borosilicate glass, has a UHMWPE (ultra-high molecular weight polyethylene) seal **107** which lubricates and is solvent resistant, and has a plunger **108**. A length of TYGON® tubing **109** connected at one end to the syringe is routed through the metal lid **105** of the jump box **106** (see Fig. 1 panel A) to a connector, and to tubing that attaches directly to the proximal end of a soda lime glass capillary tube.

Fig. 1 panel C shows a power supply **110** located inside the jump box **106** to the lower left.

Fig. 1 panel D is a photograph of the jump box **106** inside a tissue culture flow hood for sterile conditions. The metal lid **105** is in the closed position, and the outer lid **111** is in an open position. The TYGON® tube **109** for connecting to a tip extends from the metal lid **105**, to the edge **112** of the metal lid **105**.

Fig. 2 is a schematic drawing illustrating exemplary movement of the syringe plunger **108** in fluid cycling with eight cycles. The return stroke operates according to the same parameters as the execution stroke, i.e. outflow motion has the same characteristics as inflow motion. A cycle consists of an execution stroke followed by a return stroke. The position of the plunger location along the length of syringe at a time point during a cycle is represented on the ordinate, and time is represented on the abscissa. The plunger initially moves from position 0 to position 500, the load position, pauses for 200 milliseconds (ms), and moves downward to position 400, which reduces the fluid volume to less than an initial fill volume, leaving a cycling volume in the tip. After a 100 ms pause the plunger ramps from position 400 to 0. Each ramp is shown have a starting velocity, a final velocity and an acceleration from the starting to the final velocity. Each cycle has either symmetrical or asymmetrical stroke parameters such that the execution stroke and the return stroke are alike or different respectively.

Fig. 3 is a drawing and graph showing an exemplary shape of the interior surface of a tip assembly for introducing a material such as a composition in a fluid into a cell.

Fig. 3 panel A is a drawing of a three-dimensional representation of an inner surface of an exemplary tip assembly having a top or proximal opening which is located proximal to source of the material, fluid, and pressure, and a bottom or distal opening which is distal to the source of material, fluid, and pressure. The drawing shows that the tip assembly has a channel portion of a greater inner diameter and cross-section area than a distally located constriction portion. Changes in the radial dimension are not shown to scale relative to the proximal-distal dimension to emphasize the geometry of the distal end of the tip assembly. The scale bar is 200 micrometers (microns; μm).

Fig. 3 panel B is a curve showing the relationship between the length of the tip along the fluid path from the proximal to the distal opening and a radial distance from a center axis on the fluid path to an inner surface of the tip assembly, fitted using the eighth degree polynomial function characterizing the curvature of the tip assembly shown in Fig. 3 panel A. The radial distance of the inner surface of the tip assembly from a center axis on the fluid path (microns) is shown on the ordinate and the length of the tip along the fluid path (microns) is shown on the abscissa. The fluid path along the inner surface of the tip from the proximal (top) opening channel portion and constriction to the distal (bottom) opening is shown from left to right.

Fig. 3 panel C is a photograph of an exemplary tip of a tip assembly for introducing material in a fluid in a cell, with a smaller tip diameter (175-200 μm), and a constriction portion of length 250 μm proximal to the distal opening of the tip assembly. The tip assembly was made from custom fabricated glass tube having an ID of 0.88 mm, OD of 1.23 mm and wall thickness of 0.14 mm.

Fig. 3 panel D is a drawing of a three-dimensional representation of an exemplary tip as shown in Fig. 3 panel C. The drawing shows that the tip opening is narrower than the opening of the tip shown in Fig. 3 panel A. The drawing also shows a constriction portion, 250 μm long proximal to the distal end of the tip assembly. Scale bar is 500 μm.

Fig. 4 is a set of drawings and a graph showing an exemplary shape of a tip assembly and a flow-through assembly for introducing material in a fluid into a cell.

Fig. 4 panel A is a drawing of a three-dimensional representation of the inner surface an exemplary tip assembly having a top or proximal opening which is located proximal to source of the material, fluid, and pressure, and a bottom or distal opening which is distal to the source of material, fluid, and pressure. The drawing shows that the tip assembly has a channel portion of a greater inner diameter and cross-section area than a distally located constriction portion. Changes in the radial dimension are not shown to scale relative to the proximal-distal dimension

to emphasize the geometry of the distal end of the tip assembly. The scale bar is 200 micrometers μm.

Fig. 4 panel B is a curve showing the relationship between the length of the tip along the fluid path from the proximal to the distal opening and the radial distance from a center axis on the fluid path to the inner surface of the tip, fitted using the eighth degree polynomial function characterizing the curvature of the tip assembly shown in Fig. 4 panel A. The radial distance of the inner surface of the tip assembly from a center axis on the fluid path (μm) is shown on the ordinate and the length of the tip along the fluid path (μm) is shown on the abscissa. The fluid path along the inner surface of the tip from the proximal (top) opening channel portion and constriction to the distal (bottom) opening is shown from left to right.

Fig. 4C shows a flow-through assembly system. In this embodiment, the fluid enters through an entry channel, passes through a constriction, and then passes through another channel, the exit channel, before exiting the system.

Fig. 5 is a drawing of a three-dimensional representation showing an exemplary shape of a tip assembly having a body including a proximal opening **900**, attachment shoulders **901**, an attachment portion **902**, a channel portion **903**, a constriction portion **904**, and a distal opening **905**. The fluid path direction is from the proximal opening **900** to the distal opening **905** or alternatively from the distal opening **905** to the proximal opening **900**. The channel portion **903** was designed to have a greater inner diameter and cross-section area than the constriction portion **904**. The scale bar is 200 μm.

Fig. 6 is a set of photomicrographs showing primary human umbilical vein endothelial cells (HUVEC) 24 hours after a tip assembly was used to transfect cells with a plasmid encoding a fusion protein of enhanced green fluorescent protein (EGFP) and human porcine endogenous retrovirus receptor (HuPAR2). HuPAR2 protein localizes specifically in subcellular membranous compartments surrounding a cell nucleus. The cells were plated on a glass-bottom 35 millimeter Petri dish for 15 minutes for attachment to the dish, and were cultured in growth medium at 37° C.

Fig. 6 panel A shows HUVEC cells transfected using a tip assembly with vectors carrying genes encoding a fusion protein of enhanced green fluorescent protein (EGFP) and HuPAR2. The left photograph shows the cell visualized by differential interference contrast (DIC); the middle photograph shows the same cell visualized by confocal laser scanning microscopy (CLSM) of EGFP fluorescence; and the right photograph shows an overlay of the DIC photograph and the fluorescence microscope photograph. Data show that the tip assembly

effectively transfected the cell as determined by intense staining observed in perinuclear subcellular compartments indicating presence of the EGFP-HuPAR2 fusion protein in the cell. The thickness of an optical section in the fluorescence image is 0.7 μm. Thus, the image is an optical cross section through the cell showing expression of EGFP-HuPAR2 within subcellular compartments. Scale bar is 10 μm.

Fig. 6 panel B is a photomicrograph of several HUVEC cells transfected using a tip assembly with vectors carrying genes encoding the fusion protein of EGFP and HuPAR2. The cells were visualized by a combination of brightfield DIC and CLSM fluorescence and shown as an overlay of the DIC channel and the fluorescence channel. The photomicrograph shows significant perinuclear staining of GFP-HuPAR2 protein in HUVEC cells. The image for these plurality of HUVEC cells transfected with genes that encode a fusion protein of EGFP and HuPAR2 is substantially similar to the image in the photograph of the single transfected HUVEC cell (Fig. 6 panel A, left photograph).

Fig. 7 is a set of CLSM fluorescence images of primary HUVECs (human umbilical vein endothelial cells) transfected with a plasmid using the methods and systems herein, encoding a fusion protein EGFP-CDC42 (enhanced green fluorescent protein–cell division control protein 42). Scale bars are 20 μm.

Fig. 7 panel A shows EGFP-CDC42 staining observed 24 hours after transfection using laser scanning confocal microscopy. The staining was observed in the cytoplasm at the periphery compared to little or no staining in the nucleus of the cell.

Fig. 7 panel B shows EGFP-CDC42 expression as a result of transfection in a pair of daughter cells that had recently divided. Expression was observed to be concentrated in a region between daughter cells.

Fig. 7 panel C shows a larger magnification of HUVEC cells transfected with EGFP-CDC42 at standard PMT (photomultiplier tube) sensitivity. Fluorescence was observed localized to large granules.

Fig. 7 panel D shows HUVEC cells transfected with EGFP-CDC42 in which the image was scanned at a higher than standard PMT sensitivity. EGFP-CDC42 expression was observed in subcellular granules smaller in size than granules observed in panel C and located throughout the cell including the cell periphery.

Fig. 8 is a set of confocal fluorescence microscope images and a bar graph showing data obtained from primary HUVEC cells transfected using the methods and the devices herein with a 7 kb plasmid encoding an EGFP-actin fusion protein.

Fig. 8 panel A shows a cluster of primary HUVEC cells transfected with EGFP-actin. Transfection and expression of EGFP was observed localized to actin filaments of the cell cytoskeleton.

Fig. 8 panel B is an image of a primary HUVEC cells transfected with EGFP-actin showing expression of EGFP in the actin filaments of the cell.

Fig. 8 panel C is a bar graph showing EGFP-actin mRNA copy number in HUVEC cells transfected with the methods and devices herein or with an AMAXA Nucleofactor™ (Lonza Cologne GmbH, Cologne, Germany) electroporation unit. TC indicates transfection with the plasmid using the method described herein, and CT indicates a control procedure absent the EGFP-actin plasmid. AMAXA indicates data obtained with the AMAXA Nucleofactor™ electroporation unit. Transfection with the method described herein yielded significantly greater mRNA copy numbers per cell than with the commercially available AMAXA transfection device.

Fig. 9 is a line graph showing flow cytometry quantification of EGFP fluorescence of Jurkat cells transfected with an amount (2 μg, 5 μg or 10 μg per 50 μl fluid volume) of a plasmid encoding EGFP. Cells treated with 5.0 μg of plasmid showed greater EGFP fluorescence than the background fluorescence observed in control cells.

Fig. 10 is a set of confocal microscope fluorescence images of cultured primary mouse brain astrocytes transfected using methods and device herein with EGFP plasmid or Cy3-labeled miRNA. Scale bar is 10 μm.

Fig. 10 panel A is a confocal fluorescence microscope image of a few primary mouse brain astrocytes transfected with EGFP plasmid. The cell with a round shape has fluorescence intensity near background level.

Fig. 10 panel B is a confocal fluorescence microscope image of a cluster of primary mouse brain astrocytes transfected with EGFP plasmid showing cells with various levels of fluorescence intensities.

Fig. 10 panel C is a confocal fluorescence microscope image of a group of primary mouse brain astrocytes transfected with EGFP plasmid showing a cell with high fluorescence intensity surrounded by cells with lower levels of fluorescence intensities.

Fig. 10 panel D is an overlay of a confocal fluorescence microscope image of a primary mouse brain astrocyte transfected with EGFP plasmid on a brightfield image of the same cell, showing morphological features and a clear outline of the shape of the cultured cell.

Fig. 10 panel E is a confocal fluorescence microscope image of two primary mouse brain astrocytes transfected with Cy3-labeled miRNA showing internalized miRNA, which is

localized mostly in the cytoplasm as tiny round spots. The bottom left cell shows also distinct internalization of miRNA into the nucleus, as seen by two Cy3-labeled points or puncta within the confocal section through the nucleus, demonstrating that transfection resulted in entry of miRNA into the nucleus of cells.

Fig. 10 panel F is a confocal fluorescence microscope image of a group of mouse brain astrocytes transfected with Cy3-labeled miRNA showing varying levels of internalization of miRNA in different cells.

Detailed description of embodiments

Cells are surrounded by a plasma membrane comprising a bilayer of lipids and fat molecules. The integrity of the plasma membrane is affected by the electrostatic forces among the individual lipid molecules, and by interactions between the membrane and the underlying cellular cytoskeleton. Individual lipid molecules diffuse through the plane of inner leaflets and outer leaflets of the bilayer. The behavior of these lipid molecules within the bilayer is described using the fluid mosaic model of the plasma membrane that states that the lipid bilayer is charged and polar to both repel water and prevent the passage of water into and out of the cell. The bilayer serves as a barrier also for biological molecules including nucleic acids, proteins, and fats.

A pressurized fluid flow apparatus and method for transfection of cells is described by Diefenbach T. in PCT/US2010/0271040 filed March 12, 2010, WO 2010/105135 A1. Transfection of cells using Diefenbach's method results in expression of recombinant proteins in recipient cells. The apparatus has a pipe portion and a contiguous tip portion, and the apparatus is connected to a suction device at the proximal end of the pipe portion.

The invention herein provides a system, tip assembly, methods, and kits for introducing material into cells, for example for introducing a composition such as a genetic material into living cells or non-viable cells. The invention herein also provides a flow-through assembly in which the fluid enters through one channel enters a constriction portion and flows through another channel before exiting the system. In the system provided by the invention herein the tip assembly that connects to a flow device is a reusable tip assembly such that the same tip is used for multiple transfections. Optionally, a disposable tip assembly connects to a the flow device permitting a previously unused tip assembly to be used for each transfection.

Without being limited by any particular theory or mechanism of action, the methods herein use fluid dynamic technology, in combination with low Ca^{+2} conditions, passaging 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 or linear DNA in a molecular shape that is long and relatively thin. Entry of material into the cytoplasm of a cell is followed by cell transport mechanisms that convey material to a destination in the cell, for example, to the nucleus via transport along the cell cytoskeleton and entry into the nucleus through the nuclear membrane with the help of nuclear transporters. The diameter of a single DNA helix is 2 nanometer (nm). However, a circular DNA plasmid is subject to coiling and supercoiling. Openings in the cell membrane produced by the methods described herein acquire a configuration capable of permitting entry of the plasmid into the cell. Electroporation results in openings as great as 120 nm in diameter. Examples herein show that the methods herein result in openings in the membrane similar to this size, for example at least about 1 nm to about 10 nm, about 10 nm to about 60, about 60 nm to about 100 nm, about 100 nm to about 120 nm, or at least about 120 to about 140 nm.

The term "introducing" as used herein refers to any of a variety of methods for delivering a composition such as a macromolecular or a low molecular weight molecule 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. Methods for constructing vectors are shown for example in Ericsson, T. A. et al. 2003 PNAS vol. 100 (10): 6759-6764.

The term “cells” includes living cells, non-metabolizing, resting or inhibited cells, and non-reproducing cells. For methods herein, cells are suspended in a fluid or medium in a container, and a pressure differential is applied across the plasma membrane. The pressure differential is caused *inter alia* by different pressures exerted by the ionic constituents of the medium, the cytoplasm, and pressure exerted on the medium. Compressibility of the medium is a factor that affects the pressure differential. Cells are conveniently placed in various types of media, generally in aqueous media which includes biological and naturally occurring fluids such as blood or tissue. Water is relatively incompressible, and compressibility is not related to observed pressure differences imposed on the cell in aqueous media. Water temperature if constant does not affect the fluid density, which also remains constant. This pressure of an incompressible fluid is termed static pressure.

The term “passage” as used herein refers to transit of fluid and cells through at least a portion or a length of the tip assembly or the transfer of cells from a reservoir through the tip assembly into a receptacle. A single passage includes at least impelling or conveying the fluid with cells through the tip assembly. Iterations of multiple passages may include a passage cycle in which fluid is collected in the receptacle and conveyed by suction back through the tip

assembly. In related embodiments, passaging means impelling the fluid entirely through the tip assembly, including removing a suspension of cells from a receptacle into the channel portion and replacing the cells into the same receptacle, or distributing the cells through the tip assembly portion into a plurality of receptacles, or into at least one of the plurality of receptacles.

In general, ejecting the mixture under pressure during passaging 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.

Molecules in a fluid are in constant state of motion and exert pressure on the walls of a container, which is referred to as total pressure. If set into directed or ordered motion, a dynamic pressure is produced which is an additional type of pressure associated with momentum of moving molecules. Therefore motion of molecules contributes to static pressure, i.e., dynamic pressure. The addition of dynamic pressure to the static pressure results in a total pressure in a flowing system. Thus, a cell in a fluid is subject to a number of dynamic forces and pressures.

Additional fluid dynamics factors are considered in the circumstances of fluid passing in a conduit or tube into a narrowed or constricted portion. The change in pressure accompanying a change in fluid velocity through a tube with a changing cross section area is described by the Bernoulli equation which states that if a fluid flowed through a reduction in cross-sectional area of a tube an accompanying reduction in pressure and an increase in fluid velocity would be observed. The Bernoulli equation states

$$v^2/2 + gz + p/r = \text{constant}$$

in which v is the fluid flow speed at a point in the streamline, g is the acceleration due to gravity, z is elevation to a point above a reference plane with a z direction opposite to the direction of gravitational acceleration, p is the pressure, and r is the fluid density. Bernoulli's principle elucidates a reduction in tube diameter resulting in a decrease in fluid pressure, a process known as the Venturi effect.

The Venturi effect occurs as a result of satisfaction of the law of conservation of energy, from a fluid passing through a constriction increasing the fluid velocity in the constriction to conserve mass. As the velocity of the fluid increases so does the kinetic energy. Therefore a decrease in fluid pressure occurs to counteract the increase in kinetic energy.

Cells moving in a fluid-filled channel or passageway undergo an increase in speed in a constriction of the channel, in passing through a narrower diameter exit aperture. These cells in

suspension experience a sudden change from high pressure or higher pressure to a low pressure or a lower pressure. Low pressure results in temporary stretching of the cells or a temporary change in the fluidity of the plasma membrane, thereby, creating temporary holes pores, or small tears in cell membranes, permitting entry of material present in the fluid or solution surrounding the cells, including without limitation genetic materials cDNA, siRNA, miRNA. Low Ca^{+2} concentration in the fluid or solution promotes prolongation of time required for cell membrane sealing, further promoting entry of material down a 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, or other molecules in the fluid such as smaller molecules including a drug into the cells.

Examples herein utilize a tip assembly that includes a channel portion and a constriction to create pores or holes in a cellular membrane. A drawing of an exemplary three dimensional representation of the tip assembly shape is shown in Fig. 3 panel A. Without being limited by any particular theory or mechanism of action, it is envisioned that after the cells pass through the narrowing or constriction as shown for example in Fig. 3 there is a distal opening with curved edges for two-way passaging, in one embodiment an enlargement into a more distal continuation of the channel into a section having the same, larger or smaller diameter. The inside curvature of the shape shown in Fig. 3 panel A is represented in two dimensions as a cross section (Fig. 3 panel B). The cross sections of the tip assembly are parallel to the fluid flow. An exemplary curvature of the fluid flow and tip assembly shape in one embodiment characterized in two dimensions by the following equation:

$$f(x) = p1x^8 + p2x^7 + p3x^6 + p4x^5 + p5x^4 + p6x^3 + p7x^2 + p8x + p9$$

where x is a radial distance from a center axis on the fluid path to an inner surface of the tip, and the coefficients (with 95% confidence bounds in parentheses) are: p1 is 2.285 (1.388, 3.182); p2 is 3.465 (1.782, 5.149); p3 is -15.68 (-20.04, -11.32); p4 is -20.38 (-27.24, -13.52); p5 is 44.27 (36.5, 52.04); p6 is 53.96 (45.69, 62.23); p7 is -58.72 (-64.02, -53.42), p8 is -123.5 (-126.4, -120.6); and p9 is 186.5 (185.6, 187.4). Parameters applied to obtain goodness of fits were: sum of squares due to error (SSE) is 66.5, r-square (R^2) is 0.9997, adjusted R^2 is 0.9997, and root mean squared error (RMSE) 1.341.

The skilled artisan alters the degree of change in curvature in the tip assembly to change the rate at which the pressure changes during fluid flow. For example, the various channel portions are constructed having various lengths depending on a necessary volume of fluid, fluid pressure, or fluid velocity for introducing material into cells. For example, a 2 centimeter (cm)

channel portion having a 1 millimeter (mm) inner diameter at the proximal end is constructed to sufficiently accelerate 50 µl of fluid through the tip assembly. Thus, curvature is made steeper or shallower than the embodiment of curvature shown in Fig. 3 panels A and B. A shallower curvature is shown in three dimensions in Fig. 4 panel A. The cross-sectional shape of the inside curvature of the fluid path in two dimensions is shown in Fig. 4 panel B. This two dimensional curve is fitted using the eighth degree polynomial equation denoted and which is shown below:

$$f(x) = p1x^7 + p2x^6 + p3x^5 + p4x^4 + p5x^3 + p6x^2 + p7x + p8$$

where x is a radial distance from a center axis on the fluid path to an inner surface of the tip, and the coefficients (with 95% confidence bounds in parentheses) are: p1 is $-2.611e^{-16}$ ($-6.043e^{-16}$, $8.206e^{-17}$); p2 is 3.954^{e-13} (-3.195^{e-13}, 1.11^{e-12}); p3 is -1.845^{e-10} (-7.821^{e-10}, 4.131^{e-10}); p4 is 1.662^{e-08} (-2.394^{e-07}, 2.726^{e-07}); p5 is 7.537^{e-06} (-5.186^{e-05}, 6.694^{e-05}); p6 is -0.002137 (-0.009375, 0.005101); p7 is -0.003185 (-0.4114, 0.4051); and p8 = 268.6 (261, 276.3). Parameters used to obtain goodness of fit were: SSE is 31.09, R^2 is 0.9998, adjusted R^2 is 0.9997, and RMSE is 1.189.

Without being limited by any particular theory or mechanism of action, changes in curvature in the tip assembly are customized for the characteristics of the fluid used (e.g., viscosity, temperature, and molecular components) and also on the type of cell utilized as larger cells may require larger constriction sizes. Blood cells for example are only about 5 µm to about 10 µm in diameter. In contrast, *Xenopus laevis* oocytes are 1000 µm or 1 mm in diameter. Cells having a larger surface area to volume ratio (i.e., smaller cells) generally would be used in methods herein with smaller diameter constrictions and greater pressure reductions. For example, a suitable pressure reduction in the tip assemblies herein includes at least about 0.1%, about 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%,40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, or about 95%. The pressure drop resulting from the reduction in diameter of the path leading to the distal opening is dependent on volumetric flow rate and the change in diameter. For example, given a gradual reduction in diameter from 1 mm to 0.25 mm, with a flow rate of 5 ml/min of water, the pressure drop would be 0.28 millibar (mbar); with a flow rate of 10 ml/min, the pressure drop would be 5.55 mbar; with a flow rate of 10 ml/min, the pressure drop would be 5.55 mbar; with a flow rate of 50 ml/min, the pressure drop would be 27.9 mbar; with a flow rate of 100 ml/min, the pressure drop would be 55.6 mbar; and with a flow rate of 150 ml/min, the pressure drop would be 2643.4 mbar. The tip assemblies herein are used for any cell type including smaller or larger cells including animal cells, plant cells, and prokaryotic cells.

The extent of reduction in diameter of a fluid path determines the decrease in fluid pressure. The manner and degree to which the fluid pressure decreases affects the rate and amount of pore formation in the cellular membrane for a cell moving in the fluid path. Fig. 3 panel A and Fig. 4 panel A show exemplary fluid paths and channel shapes that narrow and accordingly decrease the fluid pressure and enhance pore formation in cells in the fluid path. The channel located proximal to the constriction has a uniform non-varying shape that leads to the distal narrowing or constriction. The tip assemblies envisioned herein have fluid paths or curvatures that are exemplified by the formulas above.

The tip assemblies are manufactured in various embodiments of any of a variety of suitable materials and suitable aperture sizes. The tip in related embodiments is manufactured of a polymer such as a water repellent material or coated with an agent to prevent cell adhesion to the tip assembly or droplet formation that prevents accurate dispensing of the fluid.

Methods for constructing tips include blow molding, vacuum forming, and thermoforming, are described in Smith, U.S. patent numbers 7,318,911; 6,482,362; and 6,117,394 issued January 15, 2008, November 19, 2002, and September 12, 2000; Pelletier et al., U.S. patent number 7,794,664 issued September 14, 2010; Taggart et al., U.S. patent number 6,596,240 issued July 22, 2003; and Tezuka et al., U.S. patent number 5,336,468 issued August 9, 1994. Borosilicate glass materials and methods are shown for example in Marques, U.S. patent number 7,341,966 issued March 11, 2008 and Watzke et al., U.S. patent number 5,736,476 issued April 7, 1998.

The methods and tip assemblies are suitable for incorporation into automated robotic systems, for reliable introduction of DNA into cells in a rapid and high-throughput manner. For example, the tip assemblies herein are used to transfect and to transduce cells in an automated manner to reduce contamination of cells. The tip assemblies herein are envisioned in various embodiments as attaching to manual pipettes and to automated pipettes and dispensing systems such as single-tip pipette systems and multi-tip pipette systems respectively.

Without further limiting the invention, it is here envisioned that one or more alternative mechanisms may operate to produce the observed transfection effects. Unlike most existing approaches that rely upon shock, pH, high energy or ultrasonics to affect the energetics of pore transport, these other hypothesized mechanisms rely on cell deformation per se rather than deformation arising from the cell flowing through a narrowing channel.

Repeated exposure of a cell in fluid suspension to distortion in a way that the shape of the cell alternates between spherical and oblate may cause rearrangement of the cortical cytoskeleton underlying the plasma membrane, affecting mobility of lipids or lipid rafts, or affecting the turnover of the membrane, which can influence membrane pore formation. The

fluidity of the membrane may be affected by changes in the underlying cytoskeleton (in a manner similar to that noted in red blood cells by Sventina et. al., 2004, Bioelectrochemistry, 62: 107-113; and gross cellular deformation (such as flattening of the cell) may contribute to changes in fluidity. A pressure change that might expose the cells to repeated compression/expansion independent of fluid flow (ie: a static suspension) may potentially also lead to pore formation, since pore formation itself will be dependent upon membrane fluidity. Such repetition at a rate substantially lower than existing sonic or ultrasonic frequencies, may also be visualized as resulting in a peristalsis of the cell or low-frequency pulsing that may affect the barrier energy that prevails at a pore under static cellular conditions. To optimize this effect for particular cells and transfection agents, the extent of such effects may be tested, quantified or otherwise established using a different test instrument to introduce a sudden pressure change or sequence of changes within a static fluid column. This flow-free mechanism would be relatively free of potentially destructive shear conditions which have been known to damage cells in prior art transfection protocols.

The methods herein for introducing a composition such as a genetic material into cells provide significant improvements over the prior art because the methods are rapid, reliable, economical (with no need for expensive reagents); recipient cells are characterized by very high viability and expression efficiency. The methods herein advantageously do not require viruses, for example, recombinant adenoviruses, which require biosafety level 2 due to potential infectiousness.

The methods herein are performed without use of toxic chemicals, complex procedures, or viruses. Cells that are dissociated from tissues or from the surface of a culture plate are suitable materials for use in the methods herein. Therefore methods herein are applicable to cells of a tissue obtained by dissociation, and to cell suspensions e.g., primary cells and cultured cells of a cell line.

Using the prior art method of electroporation, the highest efficacy obtained is only about 40% of recipient cells which express the nucleic acid. For adenovirus infection expression the highest efficacy obtained is about 30% to 85% and is generally lower. Gene guns typically achieve between 1% and 5% efficiency. In contrast, the methods herein resulted in visible protein expression in primary cells HUVECs (human umbilical vein endothelial cells) at transfection efficiency of about 85% to 90%.

The methods herein are applicable also to a tissue *in situ*, and to a monolayer of cells in culture. The methods herein bypass the relatively lengthy periods of time (several hours or days) required for chemical or viral vectors to express genes for functional analysis. 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 show use of a large plasmid, Enhanced green fluorescent protein (EGFP)-actin (7 kb). Data in the Example 7 show that the plasmid was introduced into cells more effectively and efficiently using the system, tip assembly and methods herein than by a conventional method using electroporation such as the AMAXA Nucleofactor ™ unit (Lonza, Cologne GmbH, Cologne, Germany). Methods in Examples herein include using a transfection agent 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 at least one passage, or a plurality of passages, for example about five to ten iterations of passages are performed within a one minute period. For example, a single passage or multiple iterations of passages, repeated passages performed at more than one time, are rapidly performed. Depending on the cell type, methods include passaging five times repeated every ten minutes within a specific period of time, for example, during a time period of about one hour, about two hours, or about five hours. For example a plurality of passages may be performed rapidly in a short period of time, for example, 10 to 50 or 50 to 100 flows in 1 minute.

In general, it was observed that a plurality of passages was sufficient to introduce material, e.g., genetic material and a drug, 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 methods herein were performed on different cell types, some of which have previously been categorized as refractory to genetic manipulation. Cell types include for example immortalized cells, actively growing cells, for example, cells in culture, primary cells and herein identified established ell lines. 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.

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

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. The material in other embodiments is a protein or a polypeptide, such as insulin, EGF, EPO, or IGF II. The material in yet other embodiments is a polysaccharide such as a dextran or amylose.

In general, the cells comprise a cell type selected from the group consisting of: epithelial cells, hematopoietic cells, stem cells, spleen cells, kidney cells, pancreas cells, liver cells, neuron cells, glial cells, Human umbilical vein endothelial cells (HUVEC), smooth or striated muscle cells, sperm cells, heart cells, lung cells, ocular cells, bone marrow cells, fetal cord blood cells, progenitor cells, peripheral blood mononuclear cells, leukocyte cells, lymphocyte cells, living postmitotic cells, physiologically inactive cells, inhibited cells, UV-inactivated cells, enucleated cells, anucleate cells, heat-killed cells, non-reproducing cells, and synthetic cells having an artificial membrane, or the like.

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. The tissue may also be derived from other vertebrate species including mouse, rat, pig, goat, horse, cow, monkey, fish and bird. Alternatively the method may also be used with living cells from invertebrate species including roundworm, flatworm, molluscs, insects, echinoderms, and the like, or with living cells from plant cells or from yeast.

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 herein by reference in their entireties.

Examples

Example 1: Design of an embodiment of the transfection system

An embodiment of the transfection system described herein is shown in Fig. 1 panels A-D. The main components of the device are a tip assembly, a computer programmable syringe pump **101**, **102** connected to the tip assembly by means of a USB converter **103** and an RS232 interface **104**, and a power supply **110**. As shown in Fig. 1 panel A the syringe pump **101**,**102** is attached to the inner side of the metal lid **105** of a jump box **106**. The USB converter **103** and the RS232 interface **104** that connects the syringe pump to an external computer are also attached to the inner side of the metal lid **105** of the jump box **106**. The hardware is not visible during use of the device in laboratory. An external computer communicating through the RS232 interface **104** and the USB converter **103** is used for controlling the syringe pump **101,102** using the data analysis software MATLAB (Mathworks, Natick, MA). Alternatively the transfection device uses a simple graphical user interface software, LabWindows/CVI Run-Time Engine 8.5.1 (National Instruments, Austin, Texas).

The position of the programmable syringe and the pump mechanism **101**,**102** on the jump box **106** right is shown in Fig. 1 B. The borosilicate glass syringe **101** has a UHMWPE (ultra-high molecular weight polyethylene) seal **107** for lubrication and solvent resistance. A TYGON® tube **109** extends through the metal lid **105** of the jump box **106** and connects to the proximal end of a soda lime glass capillary tube (the tip) through additional tubing. The capillary tube is an exemplary tip (Fig. 3 panel A) having the dimensions: inner diameter 1.1-1.2 mm, wall thickness 0.2 mm, length 75 mm.

The power supply **110** is located inside the jump box **106** to the lower left (Fig. 1 panel C). The power supply **110** is a single phase 24V, 2.5A power supply (PHOENIX CONTACT GmbH & Co. KG, Step Power, Blomberg, Germany), standard for distributor boards and flat control panels. The power supply (110) has low standby losses and high efficiency. Alternatively, a power adaptor is FSP60-11 (FSP North America), having 24V, 2.5A output. The power sources take 100-240V, 2.0A, 50-60Hz input.

As shown in Fig. 1 panel D, the jump box **106** is located in a tissue culture flow hood for sterile conditions. During use the metal lid **105** remains in the closed position and the outer lid in an open position **111**.

Example 2 : Fluid cycling parameters during transfection

This example illustrates the general flow parameters during fluid cycling through the tip of the tip assembly used in the methods and system or devices described herein.

A mixture containing cells and a composition to be introduced into the cells in a fluid is drawn into and forced out of the tip of the transfection device herein iteratively, resulting in cycles of inflows and outflows. An execution stroke followed by a return stroke constitutes a cycle. The return stroke and the execution stroke operate according to the same parameters. The programmable syringe **101,102** described in Example 1 produces the flow cycles. A schematic drawing illustrating movement of the syringe plunger **108** in an embodiment of fluid cycling is shown in Fig. 2, with time represented on the abscissa and the position along the length of syringe at which the plunger is located at that time during a cycle represented on the ordinate.

The following command sequence was used in this embodiment:

/1L14v400V900c200A500M200gL20v100v1000c1000A400M100A0G8L14v400V900c200A0R

The plunger motions and associated parameters in this command sequence proceeding from left to right are described herein. The plunger positions in the command sequence correspond to plunger positions indicated on the ordinate in Fig. 2 with the letter A added as a prefix. For example, plunger position 500 in Fig. 2 is position A500 in the command sequence. Fig. 2 illustrates eight cycles which were performed as follows:

L14 is the initial velocity ramp (acceleration), v400 is the starting velocity and V900 is the maximum velocity during this ramp. A500 is the position the syringe plunger (108) moves to initially, loading the tip. A500 is called the load position. c200 is the cutoff velocity when the plunger reaches A500. M200 is a 200 milliseconds (ms) pause at the load position. The cycling begins after a 200 ms pause, indicated by g in the command sequence. Ramps of a cycle, excluding the initial velocity ramp which has an acceleration L14, have a maximum acceleration L20, a starting velocity100 (v100), a maximum velocity 1000 (v1000) and a final or ending velocity 1000 (c1000). A downward movement to position A400 reduces the fluid volume to a less than an initial fill volume, leaving a cycling volume in the tip. M100 indicates a 100 ms pause after which there is a ramp from position A400 to A0. This represents the end of the first cycle and is indicated by G in the command sequence. The number 8 indicates that this command sequence has 8 cycles. The first cycle differs from subsequent cycles as it includes the loading step. At the end of the command sequence is a cycle with velocity ramp L14, starting velocity v400, maximum velocity V900, end position A0, and final velocity c200. The letter "R" at the end represents an execute code.

In the examples described herein similar cycles were used with cells and plasmid DNA or siRNA or miRNA.

Example 3: Design of a tip assembly

To produce a device to introduce a material into living cells, borosilicate glass tubes were precision flame-polished at one end (tip) to reduce the size of the opening. Using this device, the cells were passed through a passageway having a significantly smaller diameter and a different tip shape than has previously been used for cell passaging, with a result that the cell velocity significantly increased as the diameter of the passageway narrowed. The narrowing of the exit tip assembly of the glass tube generated the greater fluid velocities for introduction of the genetic material into the living cells. Most important, the cells necessarily had undergone a rapid change in fluid pressure as a result of the change in fluid velocity. The curvature of the inner surface of the narrowed portion of the fluid path contributed to the cell pore formation and cellular membrane openings. Further, the highly polished end was produced to prevent shear forces from forming as the cells encountered edges along the fluid path, therefore the polished end was observed to have enhanced cell viability.

Examples herein describe tip assemblies and methods of use for introducing material into a cell. Fig. 3 panel A and Fig. 4 panel A are three-dimensional representations of exemplary shapes of inner surfaces of tips having a top opening proximal to a flow device that applies a pressure such as a pump which impels a fluid, a channel portion leading to a constriction portion, and a distal opening for releasing the fluid. The channel portion has a greater inner diameter and cross-section area than the constriction portion.

Fig. 3 panel B and Fig. 4 panel B are graphs of the eighth degree polynomial function used to describe a plot of the tip assemblies of Fig. 3 panel A and Fig. 2 panel B respectively. The graphs describe a radial distance of the tip from a center axis of the fluid path on the ordinate, as a function of the length of the tip on the abscissa. The plot shows the fluid path along a length that includes: the inner surface of the tip from the proximal opening to the channel portion and constriction portion and to the distal opening.

Fig. 4C shows a flow-through embodiment of the system which has a flow-through assembly in place of a tip assembly. In this embodiment, the fluid after passing through an entry channel portion proximal to the flow device enters a constriction, and then another channel portion, an exit channel portion. The constriction instead of being at the distal end, as in the case of a tip assembly, is in between the two channel portions. The fluid is either passaged back and forth or flows through once through the constriction. Multiple constrictions of similar or dissimilar diameter or shape are envisioned in related embodiments of the flow-through assembly. The flow-through assembly differs from the tip assembly such that the fluid does not empty into a vessel; rather the fluid continues through the exit channel before exiting the system.

Example 4: Construction and adaptations of tip assemblies

Tip assemblies suitable for use in methods herein include those constructed of a variety of different types of materials, and in different sizes and shapes. Fluids having cells and materials are introduced into the tip assemblies, and the effectiveness of the tip assemblies to introduce material into cells is determined by one skill in the art of cell transfection. Adaptation of design of the tip assemblies includes producing structures with varying fluid path shapes, number of constriction portions, and by varying concentration of materials in the composition including the presence of organic and inorganic agents.

Tip assemblies can be used with various known types of reservoirs and flow devices. For example the reservoir is a centrifuge tube, a bin, a bag, or a bottle, and the flow device is a hand-held 200 μL pipette or hand pump. Each of the tip assemblies is adapted by obtaining data (e.g., pressure differentials, cell membrane porosity, transfection efficiency, presence of material in the cell and cell viability) for each of the multiple tip assemblies and each of the varying concentrations of substances in the tip assemblies.

Fig. 5 is a drawing of a three-dimensional representation of an exemplary tip assembly having a body including an opening shown at the top which is proximal, proximal opening **900**, to the reservoir and the fluid flow device, an attachment portion **902**, a tip assembly shoulder **901** for ejecting the tip assembly when attached to a flow device, a channel portion **903**, a constriction portion **904**, and a distal opening **905** shown at the bottom. The fluid path direction proceeds from the proximal opening **900** to the distal opening **905**. The channel portion **903** has a greater inner diameter and cross-section area than the constriction portion **904**. The tip assembly shoulder **901** extends laterally from an outward surface of the attachment portion **902**. A lower ejector section of an embodiment of the flow device removes the tip assembly from the flow device.

To operate, a flow device or fluid handling device in certain embodiments was attached to the tip assembly at the attachment portion. The fluid containing cells and material is drawn into the tip assembly from the distal opening through the constriction portion to the channel portion of the tip assembly. The top meniscus of the fluid surface and the end of the flow device are separated by a distance sufficient to avoid contact between the fluid with the flow device, so that different materials can be used in each tip. The fluid device wa used to impel the fluid through the channel portion to the constriction and the distal opening, and a reduced pressure in the constriction portion compared to the channel portion is achieved, forming pores in cells containing within the fluid, and a material included in the fluid is introduced into the cells. In related embodiments, a fluid containing the cells is drawn into a longer fluid path such that

sufficient fluid velocities are attained when passing through the constriction portion of the tip assembly.

The tip assemblies were evaluated under conditions and by the methods used in Examples above. The data obtained were used to determine cell viability and presence of material in the cell. Data show that the tip assemblies introduced material into cells by reducing the fluid pressure of the fluid and by increasing the membrane porosity of cells, in living cells and non-dividing cells. These tip assemblies were shown by Examples herein to be more effective, more efficient and convenient for introducing material into cells than previous methods and devices. (see Example 6)

Example 5: Expression of genetic material in human endothelial cells

Human umbilical vein endothelial (HUVEC) cells similar to most primary cells, are characterized by poor transfection rates using previously known transfection methods such as nucleofection, electroporation, and using current transfection products such as lipofectamine. The methods and tip assemblies herein were used to transfect HUVEC cells with a plasmid encoding a fusion protein of enhanced green fluorescent protein (EGFP) and human porcine endogeneous retrovirus receptor (HuPAR2), a protein that is specifically localized in perinuclear subcellular membranous compartments.

Examples herein generated an enhanced GFP (EGFP)-tagged C-terminal HuPAR-2 fusion protein (HuPAR-2/EGFP). The HuPAR-2 open reading frame (ORF) was amplified from the Topo-pCRII clone by using the primers 5'-ACGCGGTACCCAGGGGTCTACACAGTCCTTT-3' (SEQ ID NO: 1) and 5'- ACGCAGATCTAGCATCTTTGGACCTACCTAG-3' (SEQ ID NO: 2), which contain *Kpn*I and *Bgl*II restriction sites. The product was cloned into Topo-pCRII (Invitrogen Life Technologies) and excised using *Kpn*I and *Bgl*II. This fragment was cloned upstream and in-frame of the EGFP ORF in the *Kpn*I and *Bgl*II fragment of the EGFP fusion vector pEGFP-N1 (BD Biosciences CLONTECH; San Jose, CA).

HUVEC cells were grown in ATCC endothelial cell media and were contacted with a vector (35 μg/ml; 7005 base pairs) encoding a fusion protein of EGFP and HuPAR2 suspended in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO). Transfections were performed using the system and methods herein. The cells were plated on a glass bottom 35mM petri dish for 15 minutes to settle and to attach to the surface of the dish. Growth medium was added and the cells were incubated for 24 hours at 37°C and 5% CO_2.

The cells were visualized using a laser scanning confocal fluorescence microscope and contrast-enhanced version of brightfield microscopy, namely DIC (differential interference

contrast optics), and fluorescence was analyzed in ten randomly chosen microscope fields 24 hours after transfection. Total number of HUVEC cells and number of cells showing GFP-HuPAR2 fluorescence were determined, and the percentage of HUVEC cells with GFP-HuPAR2 fluorescence was calculated and compared (Table 1). Table 2 shows a statistical analysis of the data.

Photomicrographs of the HUVEC cells were analyzed using fluorescence microscopy. It was observed that an average of greater than 70% transfection efficiency was achieved for the cultured HUVEC cells. See Table 1 and Table 2. Representative DIC photomicrograph data and fluorescence photomicrograph data for a single cell and for a plurality of cells are shown in Fig. 6 panels A and B, and these data show that the cells were effectively transfected cells. Dark granules were observed by DIC in the membranous compartments surrounding the nucleus of a single HUVEC cell (Fig. 6 panel A left photomicrograph) and a plurality of HUVEC cells (Fig. 6 panel B). HUPAR2 protein localizes specifically in a perinuclear subcellular membranous compartments, and cells observed herein showed significant GFP fluorescence staining in the perinuclear subcellular compartments of the cells transfected with the GFP-HuPAR2 plasmid using methods and systems herein. Fig. 6 panel A middle photomicrograph. An overlay of the DIC photomicrograph and the fluorescence microscope photomicrograph showed that the GFP-HuPAR2 was localized specifically in the perinuclear membranous compartments. These data show HUVEC cells were successfully transfected using system, methods herein.

Table 1. HUVEC cells show GFP-HuPAR2 fluorescence in 10 randomly chosen microscope fields 24 hours after transfection

HUVEC cell number per field	GFP-HuPAR2 fluorescent cell number	percent HUVEC cells with fluorescence
10	7	70
16	12	75
10	8	80
18	17	94.4
16	8	50
13	7	53.8
20	17	85
16	12	75
9	4	44.4
15	12	80

Table 2. Statistical analysis of data shown in Table 1

	average (x)	standard deviation (sd)	standard error (se)
total number of HUVEC cells	14.3	3.68	1.16
HUVEC cells showing GFP-HuPAR2 fluorescence	10.4	4.35	1.37
percentage of total number of HUVEC cells that are Hu-PAR2 fluorescent cells	70.8	16.26	5.14

As the cells in this Example were transfected with a previously frozen vector, which was a large plasmid (7005 base pairs), the methods and tip assemblies herein were surprisingly more effective in transfecting and transducing cells with a genetic material and under circumstances that by other methods would have proven to be much less efficient.

Example: 6 Introduction of EGFP-CDC42 plasmid into HUVEC cells

The methods and system or device herein were tested by transfecting primary cells which are more delicate and usually more vulnerable to transfection than transformed cells that have been propagated in the laboratory for many years. Primary HUVECs (human umbilical vein endothelial cells) were chosen for this purpose.

HUVECs were transfected with a plasmid encoding an EGFP-CDC42 (enhanced green fluorescent protein–cell division control protein 42) fusion protein. The plasmid concentration mixed with cells was 70 µg/ml. Cells were allowed to become confluent by culturing them for two days prior to transfection, trypsinized to detach the cells from the cell culture substrate and suspended in fluid for transfection.

Transfection was performed using the parameters: fluid acceleration 6 µl/s/s, flow rate 160 µl/s, cycling volume 50 µl, and two continuous sets of cycles each set having 25 inflows and outflows of fluid through the tip impelled by a 2.5 ml syringe. A volume of 63 µl contained 500,000 cells and the plasmid.

Images of cells taken with a laser scanning fluorescence microscope 24 hours after transfection showed punctate (or spotted) subcellular EGFP-CDC42 fluorescence. The fluorescence was cytoplasmic and in the periphery of the cell, consistent with CDC42

localization, and was excluded from the nucleus (Fig. 7 panel A). Fluorescent cells in twenty randomly selected 20x magnification microscope fields were sampled. Results showed that a pooled average of 88.2% (135/153) of the cells displayed a CDC42 like EGFP fluorescence. Transfection using AMAXA Nucleofactor™ (Lonza Cologne GmbH, Cologne, Germany) electroporation unit was tested for comparison and either no transfection or lower transfection efficiency (less than 30%) was observed.

Images of transfected HUVEC cells with EGFP-CDC42 plasmid using the methods and the transfection device herein include one of EGFP expression in cells that had divided before imaging (Fig. 7 panel B). Consistent with the expression pattern of a protein with a role in cell division EGFP fluorescence was localized to a region between the daughter cells.

EGFP fluorescence of HUVEC cells transfected with EGFP-CDC42 using methods and the transfection device described herein was observed at normal PMT sensitivity and compared with that observed at higher PMT sensitivity (Fig. 7 panel C and D). At normal PMT sensitivity most of the fluorescence was observed to be localized to large granules (Fig. 7 panel C). At higher PMT sensitivity EGFP-CDC42 expression was detected in a subset of cells within numerous subcellular granules which were smaller in size (Fig. 7 panel D). Scanning at a higher PMT sensitivity was observed to offset potential autofluorescence observed in the large granules at normal PMT sensitivity. The fluorescence in the smaller granules was tested by photobleaching using high-sensitivity laser light in scanning mode and was observed to be specific to the expression of EGFP-CDC42 fusion protein.

The example above demonstrates that the methods and devices described herein led to efficient transfer of the plasmid into a primary cell and, the plasmid was transcribed into mRNA, the mRNA translated into protein and the protein was expressed with correct localization, thereby providing a successful transfection.

Example 7: Introduction of 7 kb EGFP-actin plasmid into HUVEC cells

Success in transfection can also be tested using measurement of mRNA copy number following transfection. Measurement of mRNA copy number is useful in situations such as when the fluorescence signal is faint either due to the peculiar nature of the fluorescent protein being expressed or when there is a high autofluorescence background.

Primary HUVEC cells from P5 mouse were transfected with a 7 kb plasmid encoding EGFP-actin fusion protein using methods and device described herein to observe expression of EGFP-actin in actin filament component of the cell cytoskeleton. 500,000 cells were suspended in 100 µl of phosphate buffered saline in a 1500 µl centrifuge tube to which the EGFP-actin expression plasmid at a final concentration 70 µg/ml was added and mixed with the cells.

Parameters used for transfection were: fluid acceleration, 6 μl/s/s, flow rate, 160 μl/s, cycling volume 60 μl, volume of syringe used, 2.5 ml, and sequential flow cycles consisting of three consecutive sets of 25 cycles of fluid motion through the tip. Cells were grown in culture for 48 hours and mRNA production resulting from the transfection was assessed using a method that determines the exact copy number of mRNA per cell. Shih et al. 2005, Exp Mol Pathol 79:14.

The results are shown in Fig. 8 panels A, B and C. Cells transfected with the transfected device herein are labeled as TC in Fig. 8 panel C. As a control cells were also subjected to the transfection protocol in the absence of the plasmid. These cells were also examined for EGFP-actin mRNA copy number (labeled CT in Fig. 8 panel C). Cells were also transfected using the AMAXA Nucleofector™ electroporation unit and are labeled as AMAXA in Fig. 8 panel C. Cells transfected using the AMAXA Nucleofector™ electroporator resulted in the generation of an average of 1152.75 copies of mRNA per cell against a background of only 0.08 average copies of mRNA per cells for the control cells. In contrast, cells transfected with the transfection device resulted in an average of 3652.66 copies of EGFP-actin mRNA per cell (Fig. 8 panel C). In another experiment performed under similar conditions, comparable average mRNA copy numbers were observed (1152.75 copies of mRNA per cell for transfection with AMAXA Nucleofector™ electroporator versus 3853.95 copies of mRNA per cell for transfection with the transfection device described herein). These results show that transfection using the transfection device described herein resulted in dramatically increased copy numbers of EGFP-actin mRNA per cell and the increase was reproducible.

Thus mRNA copy number measurements also confirmed that methods and devices described herein are useful to achieve efficient transfection even in a primary cell.

Example 8: Introduction of genetic material into Jurkat cells

Jurkat cells, a cell type known to be refractory as transfection recipients were tested for transfection efficiency with EGFP (Addgene, Cambridge, MA) using the methods and the systems herein. Approximately 250,000 cells were suspended in 50 μl of transfection medium, HBSS (Hanks Balanced Salt Solution, Invitrogen Carlsbad, CA) absent calcium or magnesium and contained 0.2% w/v EDTA (ethylenediamine tetraacetic acid). As control a mock transfected sample was transfected absent plasmid. Other transfections contained 2.0 μg (55 μl total volume with cells, 36 μg/ml), 5.0 μg (62.5 μl total volume with cells, 80 μg/ml) or 10.0 μg (75 μl total volume of cells, 130 μg/ml) of plasmid. Transfection parameters were: fluid acceleration 6 μl/s/s, flow rate 160 μl/s, cycling volume 60 μl using the 2.5 ml syringe and continuous cycling consisting of 25 inflows and outflows through the tip. Cells were grown in

culture for 48 hours and EGFP fluorescence (in FL1-H channel) was quantified using flow cytometry (Fig. 9).

It was observed that 10% of the cells showed 50 to 90% of maximum intensity of EGFP fluorescence. Data obtained from cells treated with 5.0 μg of plasmid showed EGFP fluorescence higher than the background level observed in the control transfection absent plasmid. Fluorescence intensities observed using either 2.0 or 10.0 μg of plasmid were at the background level.

Example 9: Transfection of primary mouse brain astrocytes using the transfection system herein

The methods and transfection system or device described herein, with some modifications were used to test transfection of another type of primary cells.

Primary astrocytes (1 to 2 million cells) were isolated from mouse brain and suspended in DMEM culture media (Dulbecco's modified Eagle medium, Invitrogen, Carlsbad, CA) and kept in a cell culture incubator (humidified, 37 °C and 5% CO_2) until transfection. For transfection cells were centrifuged and resuspended in 100 μl of HBSS (Ca^{2+}/Mg^{2+} free) containing 20 μg/ml of EGFP control plasmid (Invitrogen, Carlsbad, CA) or 20 μg/ml of Cy3-labeled miRNA (AM17011, 25 base pair designed RNA oligonucleotide, Ambion, Austin, TX).

The cells were transfected using a tip prepared as follows. A custom fabricated flint glass tube having an ID of 0.88 mm, an OD of 1.23 mm and with wall thickness of 0.14 mm was heated to obtain the curvature as described herein. A further additional microchannel, 250 μm long with a narrowing almost cylindrical portion nearest to the opening of the tip was added to the configuration of the tip. The tip culminated in a small tip opening, diameter 175-200 μm. (Fig. 3 panels C and D).

Transfection with EGFP plasmid was performed using a tip, 200 μm diameter, and transfection with Cy3-labeled miRNA was performed using a tip, 175 μm diameter.

The mixture of cells and the EGFP plasmid or the miRNA was passaged through the tip iteratively for 60-100 times using the methods and transfection device described herein. The mixture was drawn into the tip in a cycling volume of 60-80 μl. In view of the smaller diameter of the tip opening used in this transfection, cycling parameters were modified from those used in Examples 6-8, described generally in Example 2. Only the inflow parameters were changed. Inflow velocity was reduced to 50-80 μl/s and acceleration was reduced to 1-4 μl/s in order to permit unimpeded entry of cell suspension into tip without clogging. Thus assymetric cycles of slower inflow and maximum outflow velocity (240 μl/s) were utilized. After transfection cells were cultured and incubated in a cell culture incubator for three days, then fixed and mounted on glass slides for imaging using a confocal microscope.

Transfection of the primary astrocytes using a tip assembly with narrower tip opening and containing an additional microchannel at the proximal end (Fig. 3 C and D) led to successful transfer of EGFP plasmid or the Cy3-labeled miRNA into the cells. Representative images observed 3 days after transfection are shown in Fig. 10 panels A-F. Panels A and B show cells with varying levels of EGFP fluorescence intensities. Panel C shows a single bright fluorescent cell among a cluster of cells with fluorescence intensities equal to or only slightly higher than background fluorescence. Fig. D is an image showing an overlay of a fluorescence image of a transfected primary astrocyte over a brightfield image of the same cell. The image shows a highly fluorescent transfected cell spread out on a culture substrate having a morphology characteristic of primary mouse brain astrocytes.

Transfection efficiency measured one day after transfection by observing cells having fluorescence above background was determined to be 13.8% (237 cells from 5 different microscope fields). The efficiency was observed to increase to 29.8% three days after transfection (188 cells from 13 different microscope fields).

Panels E and F show transfected primary mouse brain astrocytes that have either a moderate level (panel E) or a high or background level (panel F) of internalized Cy3-labeled miRNA in the cytoplasm of the cells. The miRNA is excluded from the nucleus. The labeled miRNA appear as tiny round fluorescent spots. Transfection efficiency measured 3 days after transfection by observing cells that had fluorescence above background was determined to be 40.9% (41 cells from three microscope fields).

This example further demonstrates the applicability of the methods and transfection device described herein for successful transfection of primary cells, thereby extending the utility of the methods and devices herein.

Example 10: Expression of genetic material in cells using systems and methods herein.

The system and methods described herein are used to introduce genetic material into cells selected from the group consisting of: epithelial cells, hematopoietic cells, stem cells, spleen cells, kidney cells, pancreas cells, liver cells, neuron cells, glial cells, smooth or striated muscle cells, sperm cells, heart cells, lung cells, ocular cells, bone marrow cells, fetal cord blood cells, progenitor cells, peripheral blood mononuclear cells, leukocyte cells, lymphocyte cells, living postmitotic cells, physiologically inactive cells, inhibited cells, UV-inactivated cells, enucleated cells, anucleate cells, heat-killed cells, non-reproducing cells, prokaryotic cells, and synthetic cells having an artificial membrane.

A fluid containing a mixture of cells, and a plasmid encoding GFP-HuPAR2 (see Example 5) or another plasmid or mixture of plasmids, for example, a plasmid encoding a therapeutic protein alone or with a reporter plasmid encoding a fluorescent protein such as EGFP, is drawn into and forced out of the tip of the transfection device herein iteratively generating fluid cycles. (Example 2 and Figure 2). The fluid cycling results in transfection. The system uses tip assemblies described in Examples 3-4 and Figures 3-4. After transfection, growth medium is added and cells are incubated at 37° C and 5% CO_2 for 12-24 hours. The cells are then assessed for viability and further visualized using laser scanning confocal fluorescence microscope and contrast-enhanced brightfield microscope. Cells are also transfected in the absence of a plasmid to provide a negative control for transfection.

What is claimed is:

1. A system for introducing a composition in a fluid into cells, comprising;

a flow device that generates at least one of a positive pressure and a negative pressure for impelling the fluid; and,

a tip assembly for passaging a mixture of the cells and the fluid comprising: an attachment portion that proximally connects to the flow device, a channel portion contiguous to and distal to the attachment portion and the flow device, a constriction portion contiguous with the channel portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, wherein a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, wherein increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, whereby the system introduces the composition into the cells through the membrane.

2. The system according to claim 1, further comprising a receptacle adjacent to the opening for receiving the fluid passaged through the tip assembly.

3. The system according to claim 1 or claim 2, characterized in that:

the tip assembly comprises at least one selected from the group consisting of: a glass, a metal, a plastic, a polymer, a nano-based composition, a composite material comprising at least two different types of substances, and the like; and is further optionally characterized by one or more properties selected from among:

the flow device comprises at least one selected from the group consisting of: a syringe, a plunger, a bulb, a diaphragm, and a compressor; or,

operation of the flow device comprises control of at least one selected from the group of: positive fluid pressure; negative fluid pressure; flow velocity; flow acceleration; mass flow rate; initial velocity ramp; starting velocity; maximum velocity; starting position; cutoff velocity period of time the fluid is under pressure generated by the flow device; temperature; and period of time the fluid is held in the channel portion, constriction portion, or both; or,

the flow device is optionally further characterized by at least one of: a flow velocity generated along the length of the tip assembly, wherein the flow velocity is optionally selected from: about 0.1 centimeter per second (cm/s) to about 1 cm/s, about 1 cm/s to about 5cm/s,

about 5cm/s to about 15 cm/s, and about 15 cm/s to about 20 cm/s; a mass flow rate generated by the flow device, wherein the mass flow rate is optionally selected from: about 0.01 milliliter per minute (ml/min), about 1 ml/min, about 10 ml/min, about 25 ml/min, about 50 ml/min, about 100 ml/min, and about 150 ml/min; and an acceleration generated by the flow device in the tip assembly of about 0.6 μl/s/s, about 1 μl/s/s, about 6 μl/s/s, about 10 μl/s/s, about 12 μl/s/s, about 24 μl/s/s, about 36 μl/s/s, about 48 μl/s/s, and about 60 μl/s/s.

4. The system according to any of claims 1-3, wherein the flow device is controlled or operated manually.

5. The system according to any of claims 1-3, wherein control and operation of the flow device is at least one selected from the group of: automated, electromechanical, and programmable.

6. The system according to any of claims 1-5 further comprising at least one selected from the group of: a power source; a connector for interacting with a user interface, a computer, a hand-held device, a transmitter, or a display; a conduit for connecting the flow device to the tip assembly; a sensor, a valve; and a housing.

7. A tip assembly for introducing a composition in a fluid into cells comprising:

an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid;

a channel portion contiguous to and distal to the attachment portion and the flow device;

a constriction portion contiguous with the channel portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, wherein a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, wherein increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, whereby the tip assembly introduces the composition into the cells through the membrane.

8. The tip assembly according to claim 7, characterized in that:

the tip assembly is selected from at least one of: disposable, modular, transparent, and translucent; and is further optionally characterized by one or more properties selected from among:

at least one of the attachment portion, the channel portion, and the constriction portion comprises a substance selected from the group of: a glass, a metal, a plastic, a polymer, a nano-based composition, a composite material comprising at least two different types of substances, and the like; or,

an inner diameter or outer diameter of the attachment portion of the tip assembly fits the flow device in a male to female arrangement respectively; or,

the channel portion cross sectional area or the constriction portion cross sectional area is bounded by a continuous circle, an ellipse, a rectangle or a regular polygon such as a square and the circle polygon or other cross sectional shapes are continuous or discontinuous, and wherein the distal end of the constriction portion comprises an inner diameter that is less than or substantially equal to an inner diameter of the opening; or,

the tip assembly contains a volume which is: about 2 microliters (μl), about 20 μl, about 50 μl, about 200 μl, about 400 μl, about 500 μl, about 1 milliliter (ml), about 5 ml, and about 10 ml, and wherein the channel portion inner diameter is about 1.0 millimeter (mm) to about 10.0 mm, and the diameter of the constriction inner diameter is about 0.05 mm to about 2.0 mm, wherein the constriction inner diameter is smaller than the channel portion inner diameter.

9. The tip assembly according to claim 7 or claim 8, wherein the channel portion curvature and constriction inner curvature characterize a fluid path for the fluid flowing through the tip assembly, wherein the channel portion curvature and constriction inner curvature necessary for introducing the composition into the cells comprises a formula represented in two dimensions is

$$f(x) = p1x^7 + p2x^6 + p3x^5 + p4x^4 + p5x^3 + p6x^2 + p7x + p8$$

wherein x is a radial distance from a center axis on the fluid path to an inner surface of the tip and coefficients with 95% confidence bounds in parentheses comprise: p1 is $-2.611e^{-16}$ ($-6.043e^{-16}$, $8.206e^{-17}$), p2 is 3.954^{e-13} (-3.195^{e-13}, 1.11^{e-12}), p3 is -1.845^{e-10} (-7.821^{e-10}, 4.131^{e-10}), p4 is 1.662^{e-08} (-2.394^{e-07}, 2.726^{e-07}), p5 is 7.537^{e-06} (-5.186^{e-05}, 6.694^{e-05}), p6 is -0.002137 (-0.009375, 0.005101), p7 is -0.003185 (-0.4114, 0.4051), and p8 = 268.6 (261, 276.3), or

$$f(x) = p1x^8 + p2x^7 + p3x^6 + p4x^5 + p5x^4 + p6x^3 + p7x^2 + p8x + p9$$

wherein x is a radial distance from a center axis on the fluid path to an inner surface of the tip and coefficients with 95% confidence bounds in parentheses comprise: p1 is 2.285 (1.388, 3.182), p2 is 3.465 (1.782, 5.149), p3 is -15.68 (-20.04, -11.32); p4 is -20.38 (-27.24, -13.52); p5 is 44.27 (36.5, 52.04); p6 is 53.96 (45.69, 62.23); p7 is -58.72 (-64.02, -53.42), p8 is -123.5 (-126.4, -120.6), and p9 is 186.5 (185.6, 187.4).

10. The tip assembly according to any of claims 7-9, further comprising a shoulder extending laterally from of an outward surface of the attachment portion, for example a lower ejector section of the flow device removes the tip assembly from the flow device.

11. A method for introducing a composition in a fluid into cells, comprising:

contacting the cells in a reservoir with the fluid comprising the composition;

inserting a tip assembly into the reservoir, wherein the tip assembly comprises: an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid; a channel portion contiguous to and distal to the attachment portion and the flow device; a constriction portion contiguous with the channel portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, wherein a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, wherein increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells; and,

passaging a mixture of the cells and the fluid with the composition at least once through the tip assembly using a flow device, whereby passaging the mixture introduces the composition into the cells through the membrane.

12. The method according to claim 11, further characterized in that:

wherein passaging the mixture further comprises redirecting the mixture to the reservoir, or wherein passaging the mixture further comprises dispensing the mixture into a receptacle; and is further optionally characterized by one or more properties selected from among:

the cells comprise a population of a plurality of living cells, optionally eukaryotic cells, wherein the method further comprises observing maintenance of cell viability, the cell viability

is not substantially reduced and comprises at least about: 1%, 10%, 90%, or 95% of control cells not passaged through the tip assembly; or,

the fluid comprises a Ca^{+2} concentration less than about 200 nanomolar (nM), about 150 nM, about 100 nM, or about 75 nM, and wherein the fluid comprises a Mg^{+2} concentration less than about 200 nM, about 150 nM, about 100 nM, or about 75 nM; or,

the composition comprises a DNA or an RNA, wherein the DNA is selected from the group of: native DNA, synthetic DNA, cDNA, dsDNA, ssDNA, and wherein the RNA is selected from the group of: mRNA, tRNA, rRNA, siRNA, RNAi, miRNA, and dsRNA, or a portion thereof, and the method further involves assaying transfection of the cells.

13. The method according to claim 11 or claim 12, further comprising after passaging,

observing localization of the composition to at least one subcellular compartment selected from: a nucleus; a mitochondrion; a Golgi body; a chloroplast; an endoplasmic reticulum; a chromoplast; an endosome, a vesicle, a lysosome, an axon; a cytoplasmic membrane; a nuclear membrane; a cytoskeleton, and a cytoplasm, wherein observing the localization further comprises at least one of: visualizing the composition with a detectable marker selected from the group consisting of: detectable, fluorescent, colorimetric, enzymatic, radioactive; and quantifying directly the product of the composition that entered the cell from the group consisting of: mRNA, DNA, RNA, and protein; and is further optionally characterized by,

the cells comprise a cell type selected from the group consisting of: epithelial cells, hematopoietic cells, stem cells, spleen cells, kidney cells, pancreas cells, liver cells, neuron cells, glial cells, human umbilical vein endothelial cells (HUVEC), smooth or striated muscle cells, sperm cells, heart cells, lung cells, ocular cells, bone marrow cells, fetal cord blood cells, progenitor cells, peripheral blood mononuclear cells, leukocyte cells, lymphocyte cells, living postmitotic cells, physiologically inactive cells, inhibited cells, UV-inactivated cells, enucleated cells, anucleate cells, heat-killed cells, non-reproducing cells, and synthetic cells having an artificial membrane or the like.

14. The method according to any of claims 11-13 further comprising after passaging, centrifuging the mixture to obtain a cell pellet and a supernatant, removing the supernatant, adding culture medium to the reservoir, re-suspending the cell pellet in the medium, and culturing the cells.

15. The method according to any of claims 11-14 further comprising applying to the mixture before, during or after the method at least one selected from the group of: an electric field, a light or radiation comprising a wavelength, and a sound pulse.

16. A kit for introducing a composition in a fluid into cells comprising:

a tip assembly for passaging a mixture of the cells and the fluid comprising: an attachment portion open to the atmosphere that proximally connects to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid; a channel portion contiguous to and distal to the attachment portion and the flow device; a constriction portion contiguous with the channel portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than a channel portion inner diameter and a channel portion cross sectional area, wherein a distal end of the constriction portion comprises an opening for ejecting or drawing the fluid, wherein increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the channel portion affects membrane permeability of the cells, whereby the tip assembly introduces the composition into the cells through the membrane; and,

a container.

17. The kit according to claim 16, further comprising instructions for use including: contacting the cells in a reservoir with the fluid comprising the composition; inserting the tip assembly into the reservoir; contacting the attachment portion of the tip assembly to a flow device that generates at least one of a positive pressure and a negative pressure; and passaging a mixture of the fluid and the composition at least once through the tip assembly using the flow device, whereby passaging the mixture affects membrane permeability of the cells and introduces the composition into the cells.

18. The kit according to claim 16 or claim 17, further comprising a reservoir; and optionally further comprising at least one of: a transfection agent, a buffer, and a medium specific for a type of cell, cell line or strain.

19. A flow-through assembly for introducing a composition in a fluid into cells comprising:

an entry channel portion open to the atmosphere that connects to and is proximal to a flow device that generates at least one of a positive pressure and a negative pressure for directing the fluid into the entry channel portion;

a constriction portion contiguous to and distal to the entry channel portion and the flow device;

an exit channel portion contiguous with the constriction portion and distal to the flow device and the constriction portion, wherein a constriction portion inner diameter and a constriction portion cross sectional area are smaller than inner diameters and cross sectional areas respectively of the entry channel portion and the exit channel portion respectively, wherein a distal end of the exit channel portion comprises an opening for ejecting or drawing the fluid, wherein the fluid flows through the constriction portion in either direction, and wherein an increased fluid velocity and a decreased pressure in the fluid in the constriction portion compared to velocity and pressure in the entry or the exit channel portion affects membrane permeability of the cells, whereby the flow-through assembly introduces the composition into the cells through the membrane.

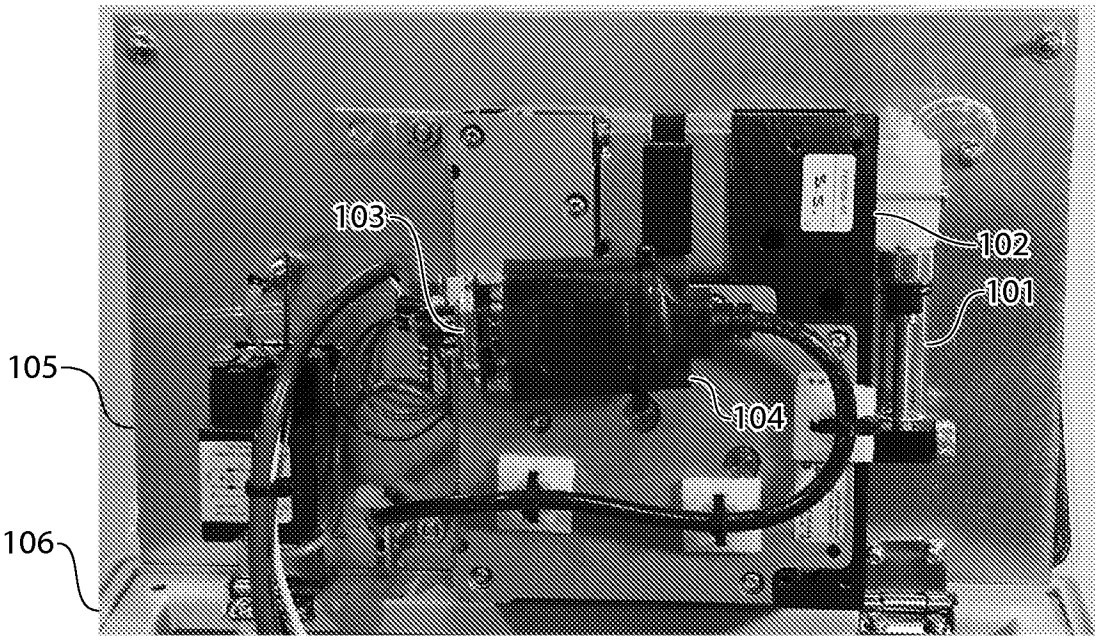

Fig. 1A

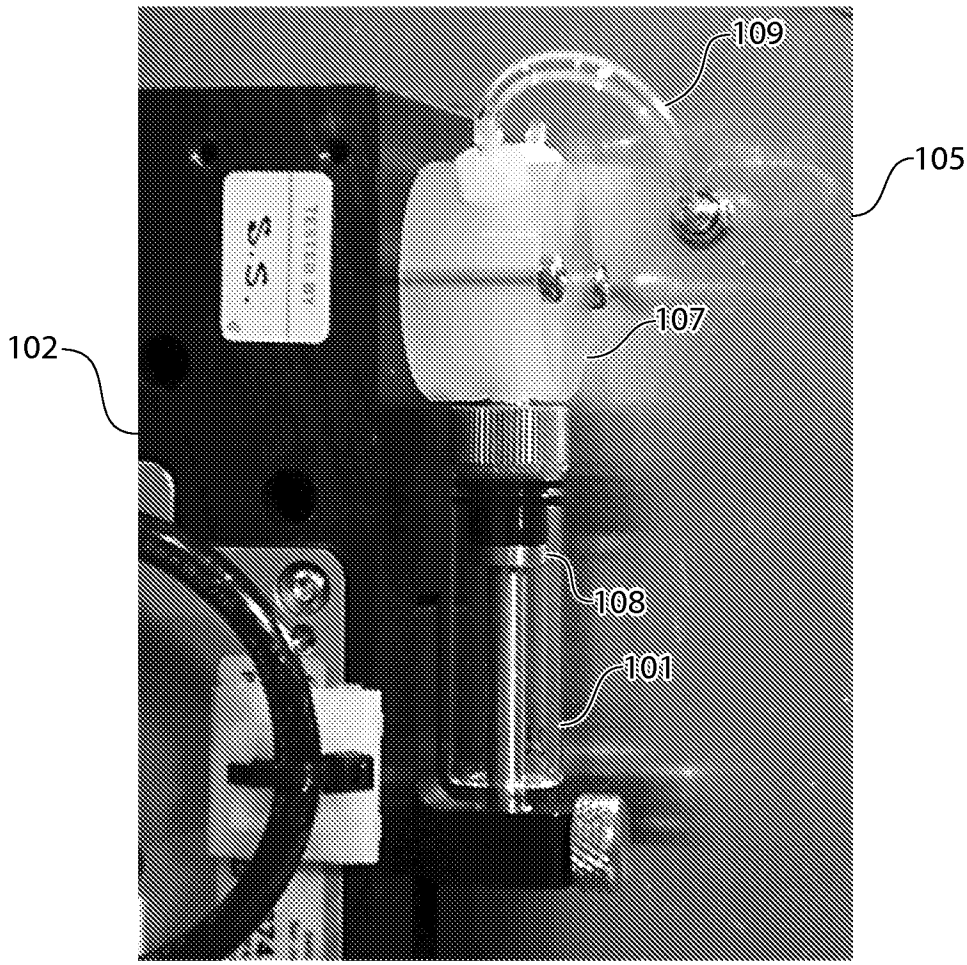

Fig. 1B

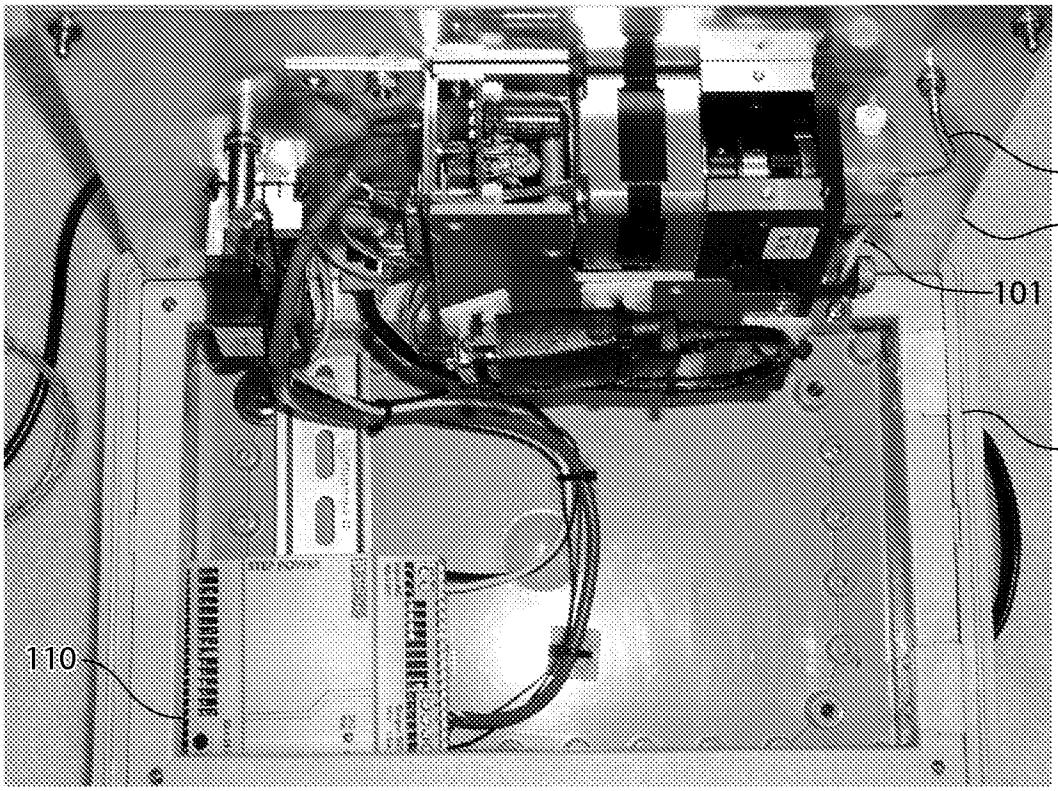

Fig. 1C

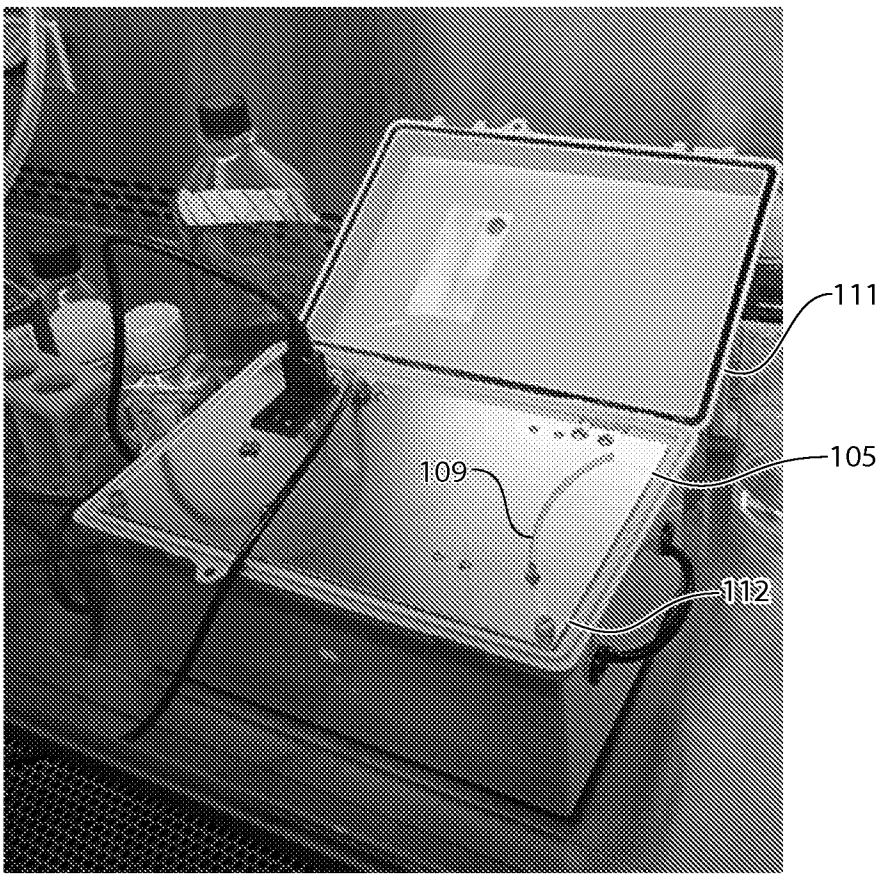

Fig. 1D

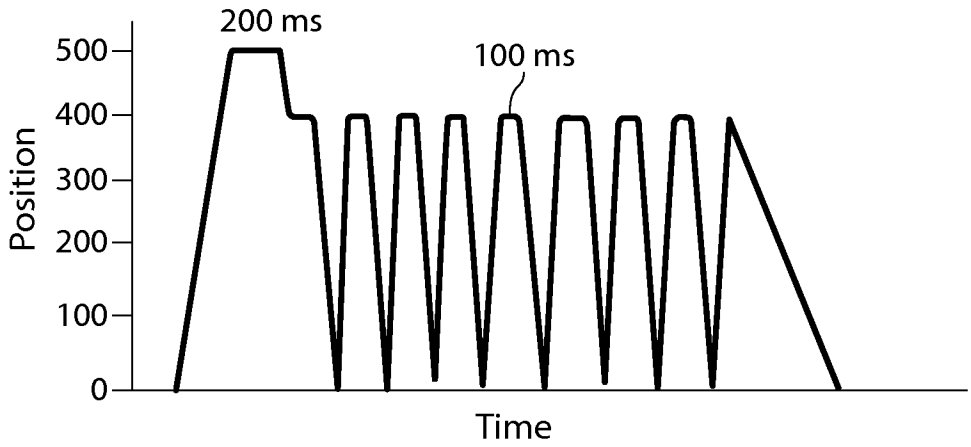

Fig. 2

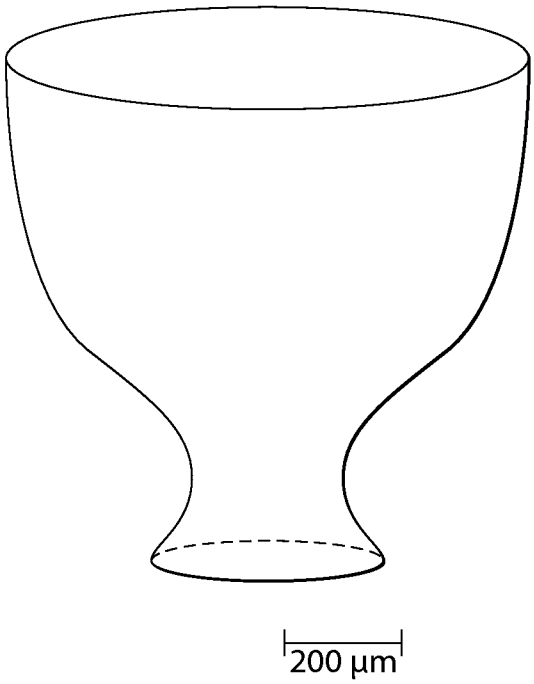
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Fig. 3A

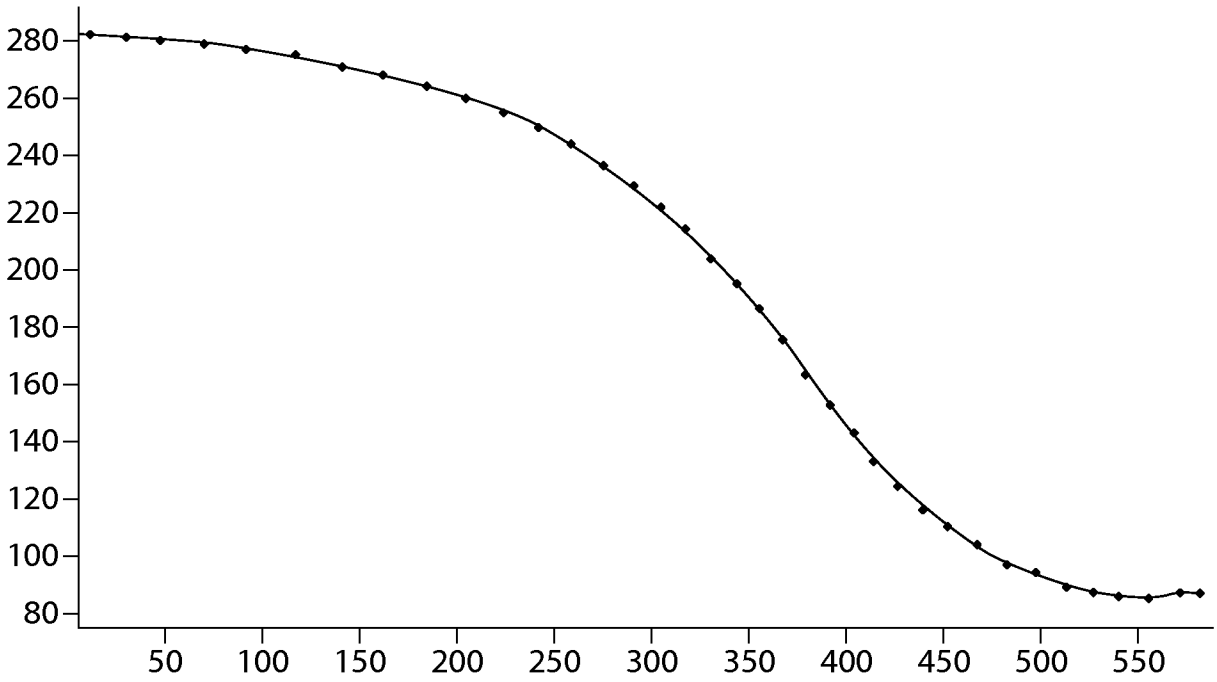
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Fig. 3B

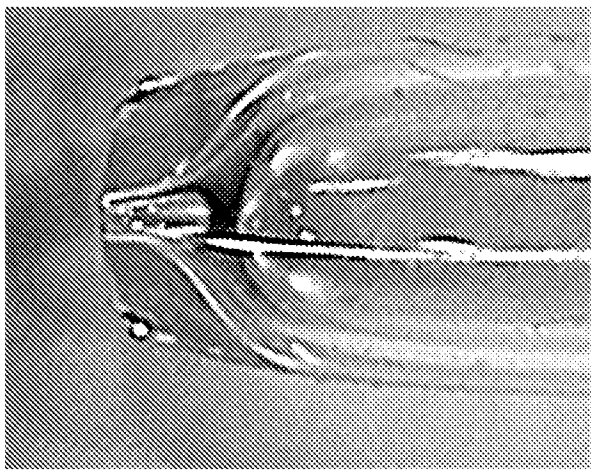

Fig. 3C

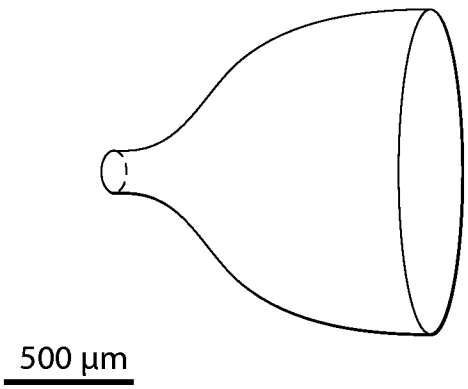

Fig. 3D

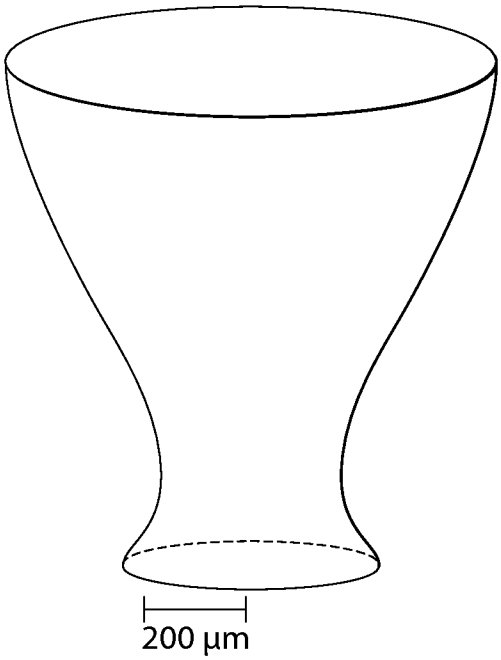
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Fig. 4A

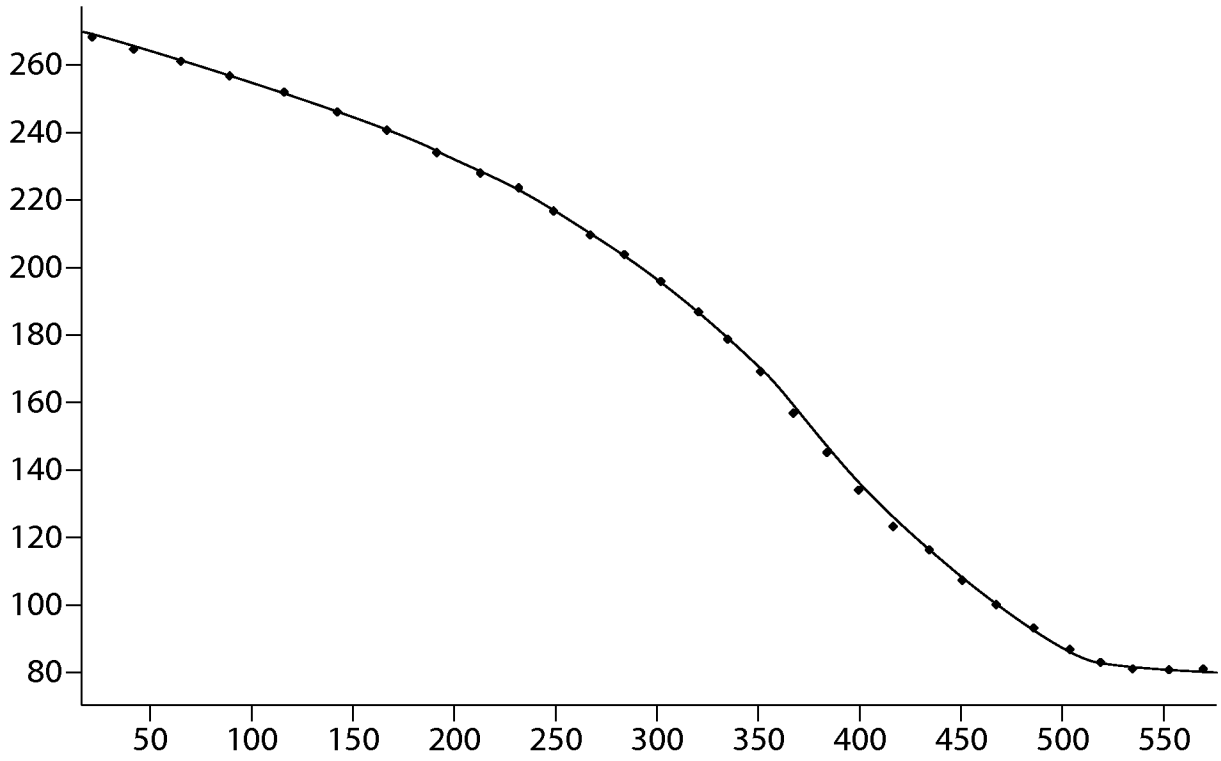
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Fig. 4B

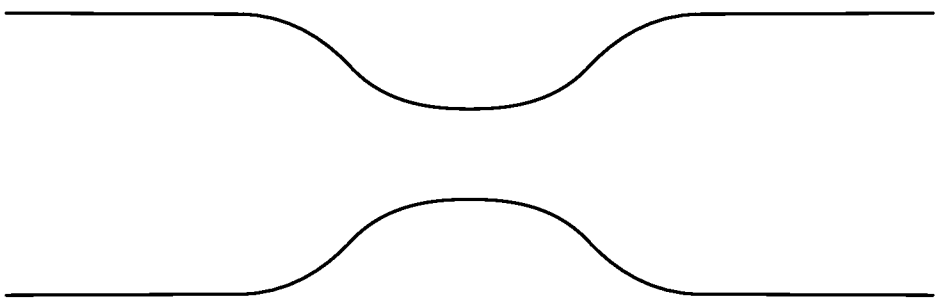

Fig. 4C

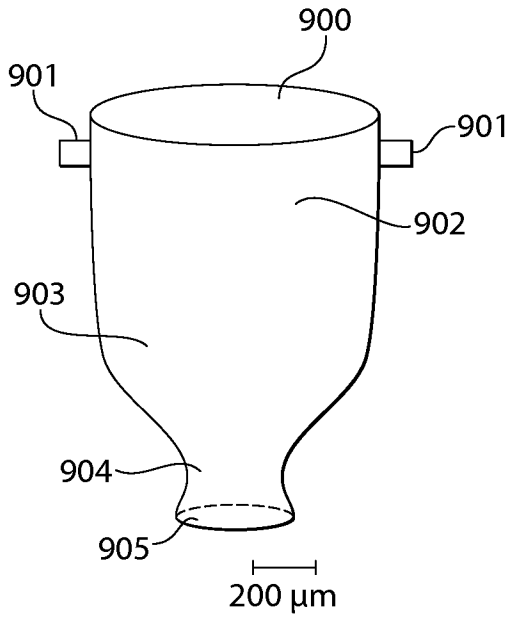

Fig. 5

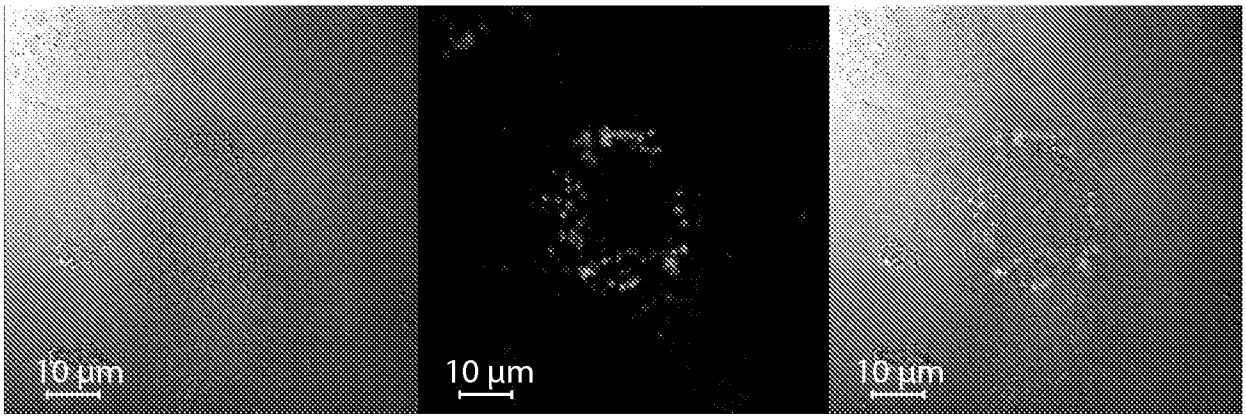

Fig. 6A

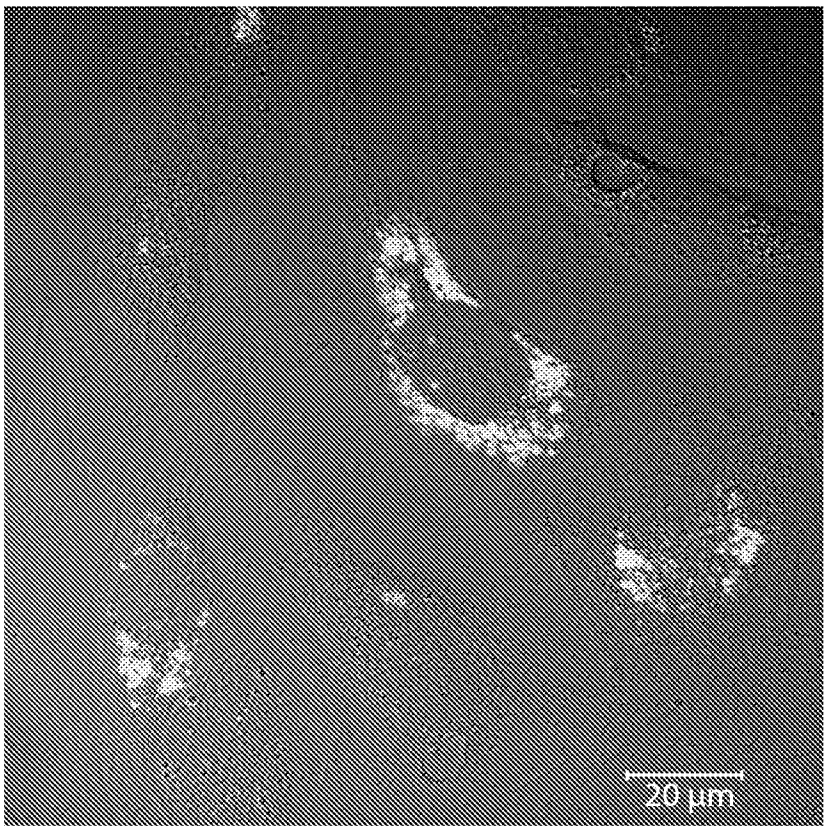

Fig. 6B

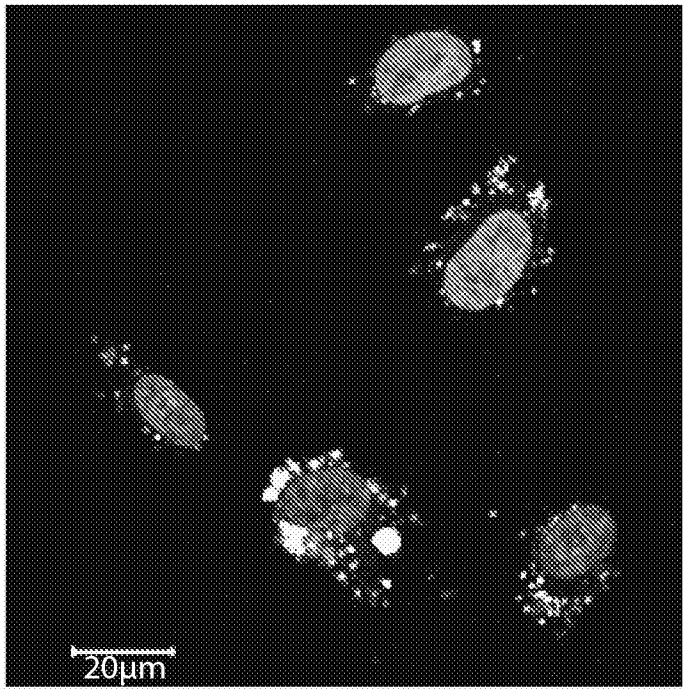

Fig. 7A

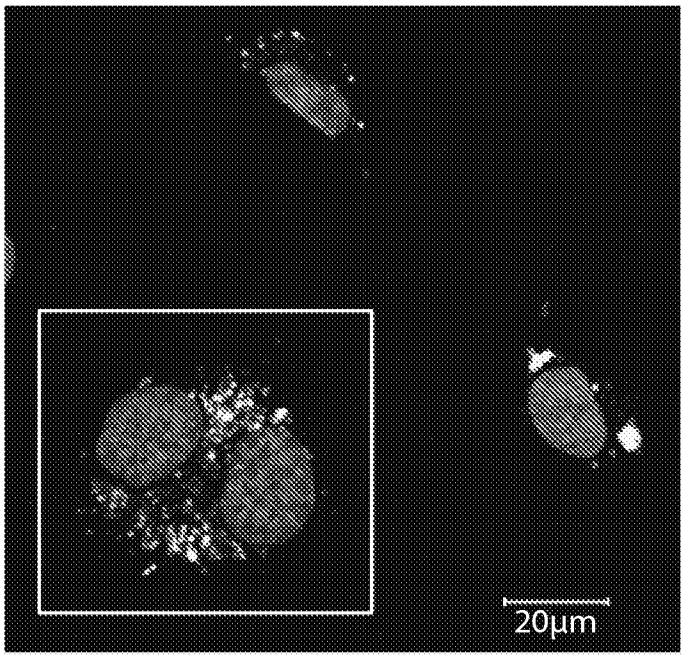

Fig. 7B

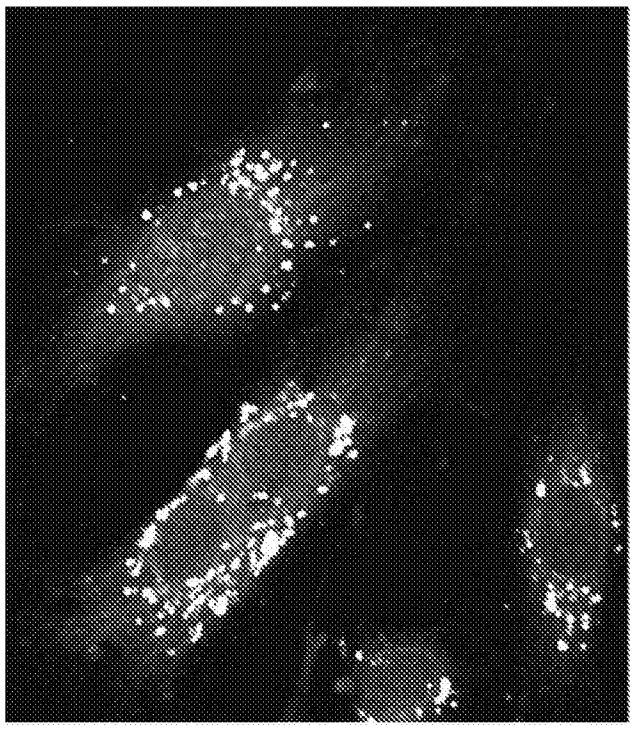

Fig. 7C

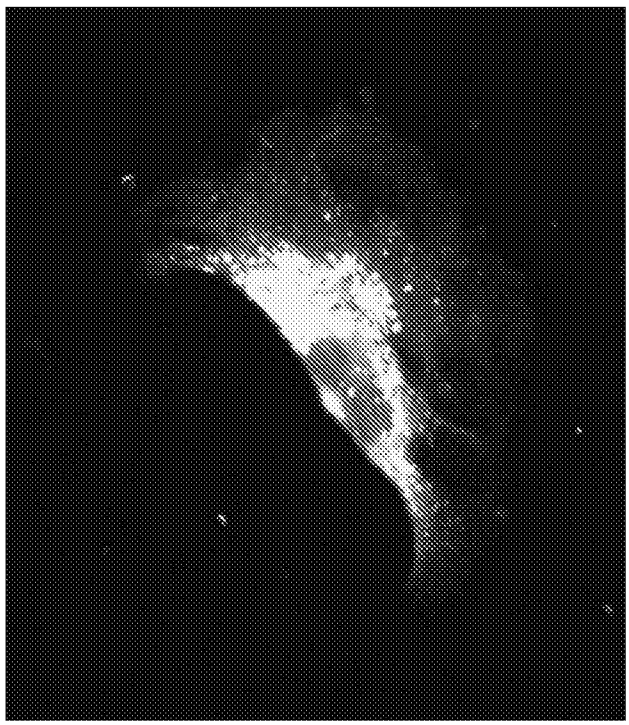

Fig. 7D

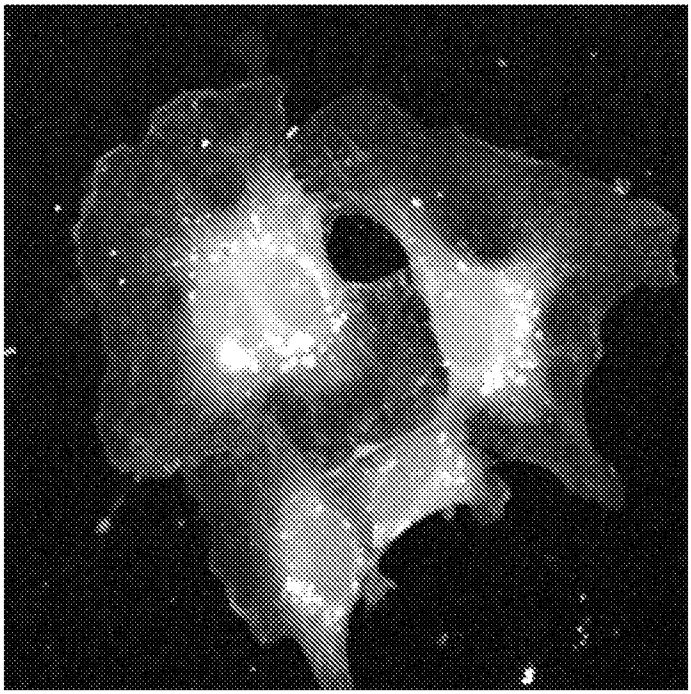

Fig. 8A

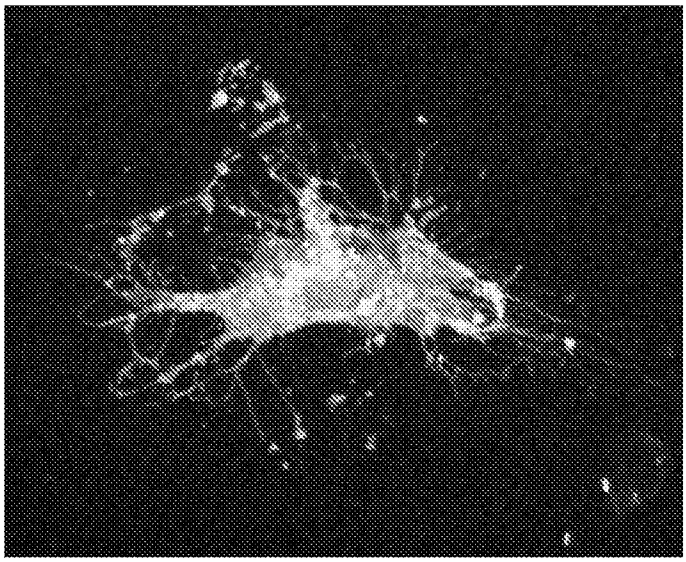

Fig. 8B

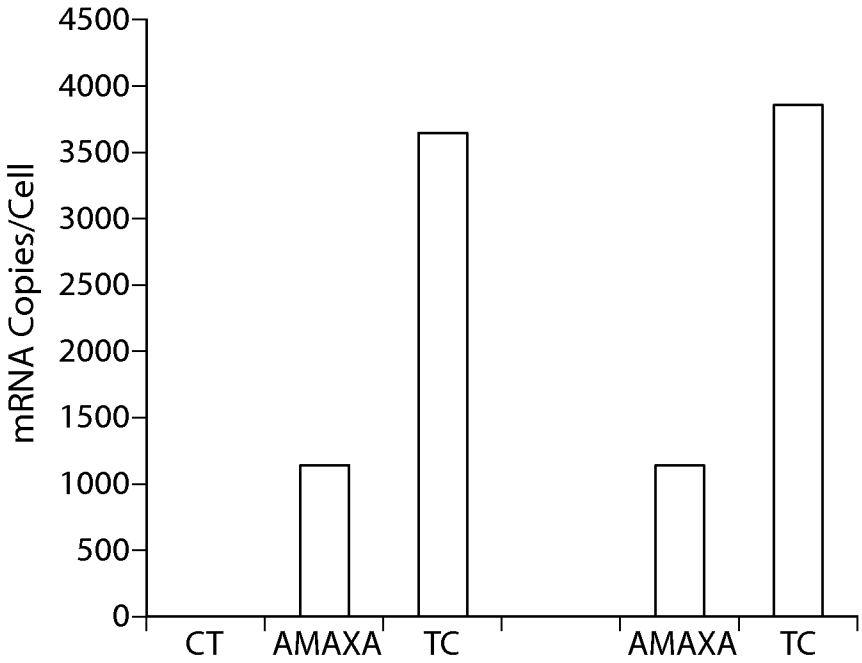
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Fig. 8C

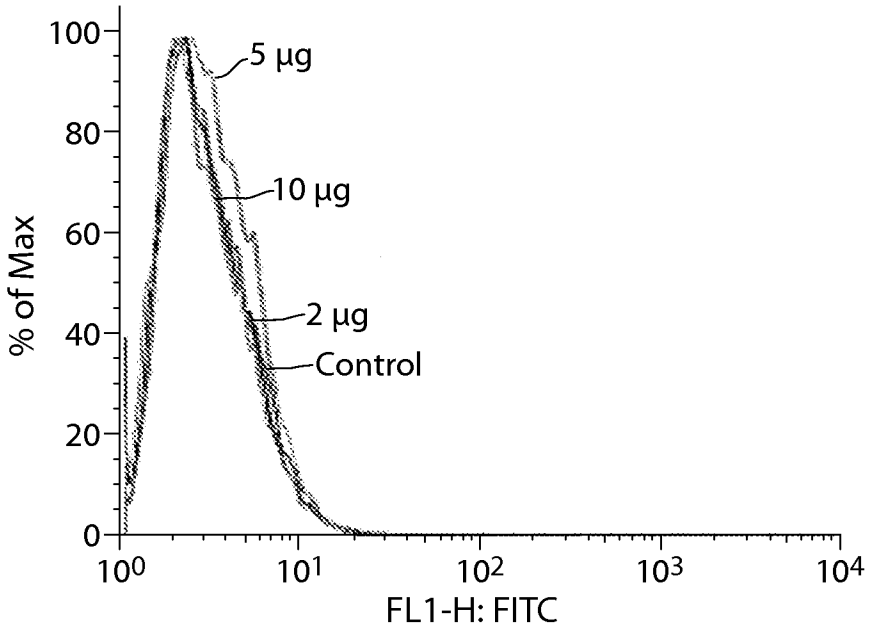
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Fig. 9

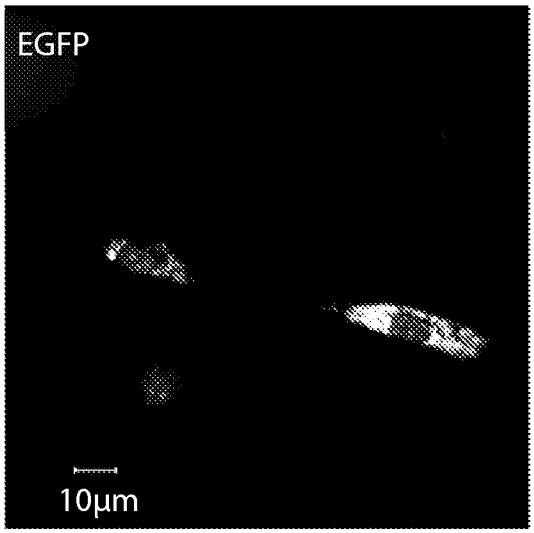

Fig. 10A

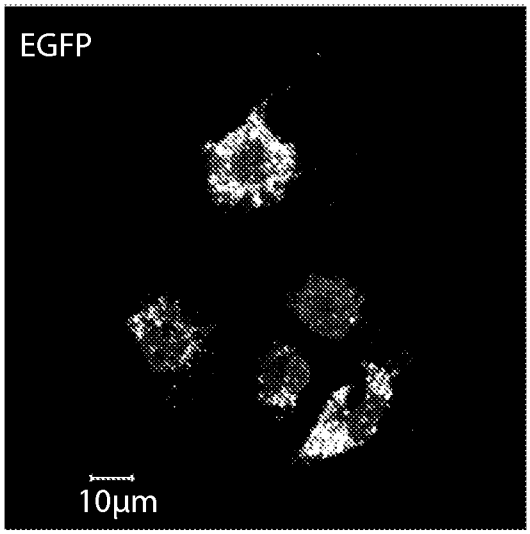

Fig. 10B

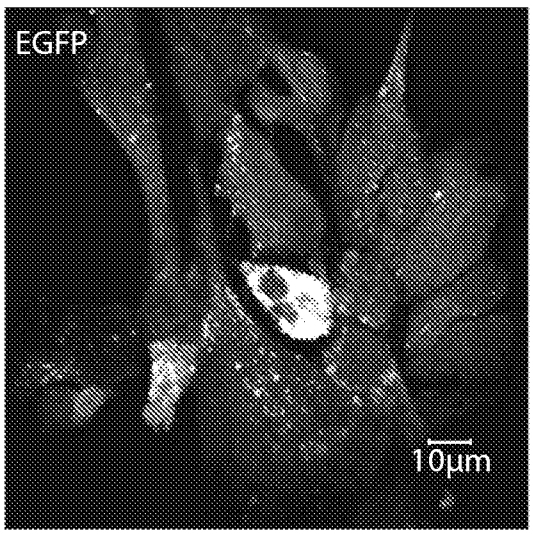

Fig. 10C

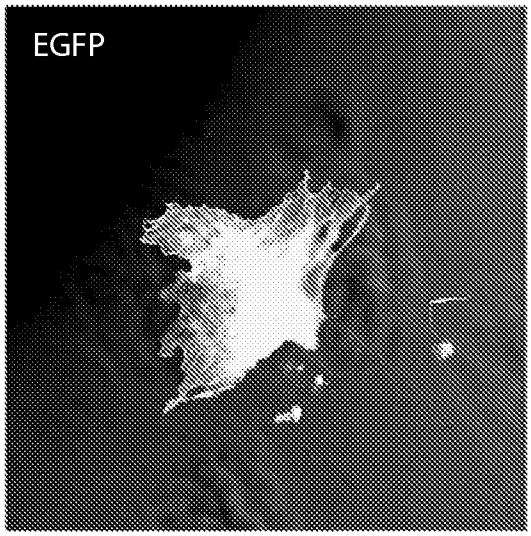

Fig. 10D

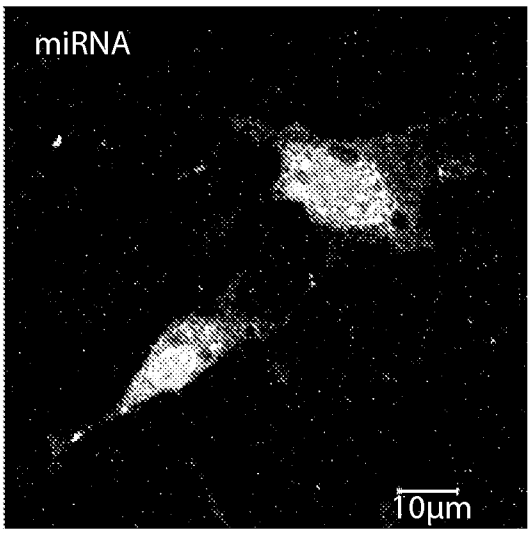

Fig. 10E

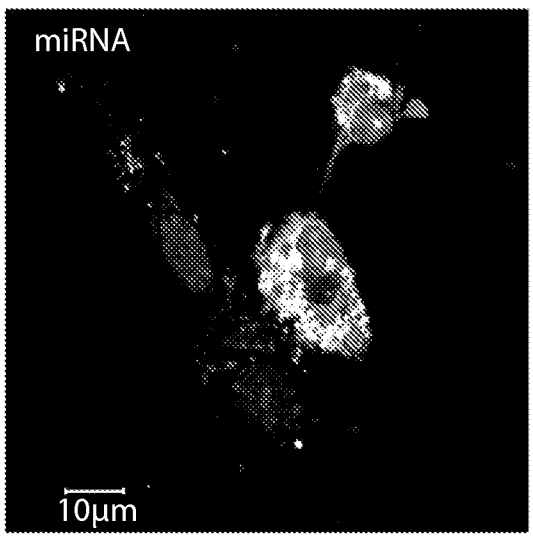

Fig. 10F