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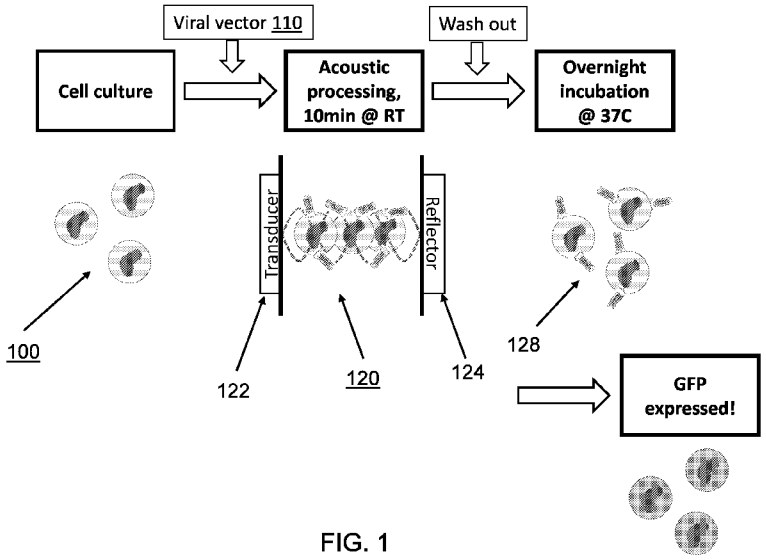


FIG. 1

(57) Abstract: Methods for introducing foreign nucleic acids into cells, such as by performing transfection / transduction, using acoustic processes are disclosed herein. The foreign DNA/RNA and the cells are co-located in a multi-dimensional acoustic standing wave, or are co-located by acoustic streaming.

[Continued on next page]

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ACOUSTIC PROCESSES FOR TRANSFECTION AND TRANSDUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 62/641,234, filed on March 9, 2018, and to U.S. Patent Application Serial No. 15/947,746, filed April 6, 2018, all of which are incorporated herein by reference in their entireties.

BACKGROUND

[0002] The present disclosure relates to methods of introducing foreign nucleic acids into cells, such as by transfection and transduction utilizing acoustic waves. Also included are cells and associated compositions produced by such methods. Such methods and compositions may be useful in cell therapy applications

[0003] Transfection and transduction are processes for intentionally introducing nucleic acids (either DNA or RNA) into cells. Transduction is performed using a viral vector such as a bacteriophage or other virus. Viruses such as adenovirus, lentivirus, or paramyxovirus are commonly used. Transfection refers to the introduction of the nucleic acids into a cell using non-viral methods. .

BRIEF DESCRIPTION

[0004] The present disclosure relates, in various embodiments, to processes for introducing foreign nucleic acids (DNA/RNA) into cells, such as by performing transfection or transduction of cells using acoustic waves. Very generally, the acoustic waves are used to bring the cells and the nucleic acids together, such that the nucleic acids can be transferred to the cells. An acoustic device can be used for this purpose, and such devices are described herein. Also included are cells and associated compositions produced by such methods.

[0005] Disclosed herein in various embodiments are methods for causing transfection or transduction of cells. The nucleic acids may be naked, or they may be in a viral vector. The cells and nucleic acids are placed in an acoustophoretic device, for example by placing them in a bag that is inserted into the acoustophoretic device, or by flowing a fluid mixture containing both the cells and the nucleic acids through the acoustophoretic device. The acoustophoretic device comprises: an acoustic chamber in which the cells

and the nucleic acids are placed; and an ultrasonic transducer and a reflector opposite the ultrasonic transducer, the ultrasonic transducer including a piezoelectric material that can be driven to create a multi-dimensional acoustic standing wave in the acoustic chamber. The ultrasonic transducer is driven to create an acoustic standing wave. As a result, the cells and the nucleic acids are co-located by the acoustic standing wave. Put another way, the cells and the nucleic acids are placed in close enough proximity to each other to permit reactions between each other. The acoustic standing wave may be a multi-dimensional acoustic standing wave, a planar standing wave, or a combination of both.

[0006] In some embodiments, the nucleic acids are in a viral vector. Transduction can occur when the virus attaches itself to the target cell and injects the nucleic acid into the target cell. In other embodiments, pores are opened in the cell membranes of the cells prior to co-locating the cells with the nucleic acids. Transfection can occur when the nucleic acid enters the cell, for example via the pores. The pores can be opened by electroporation, sonoporation, or by exposure to calcium phosphate.

[0007] The cells and the nucleic acids (either naked or in a viral vector) may be suspended in a fluid. Such fluids can include cell culture media, water, saline solution, and the like.

[0008] In particular embodiments, the cells are Chinese hamster ovary (CHO) cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, human cells, regulatory T-cells, helper T-cells, cytotoxic T-cells, memory T-cells, effector T-cells, gamma delta T-cells, Jurkat T-cells, CAR-T cells, B cells, or NK cells, peripheral blood mononuclear cells (PBMCs), algae, plant cells, or bacteria.

[0009] The ultrasonic transducer may be driven for a time period of about 5 minutes to about 15 minutes, although this time period can vary as desirable. The ultrasonic transducer may be driven at a frequency of about 0.5 MHz to about 20 MHz. In some embodiments, the frequency of the multi-dimensional acoustic standing wave is varied in a sweep pattern to move the cells relative to the nucleic acids.

[0010] The piezoelectric material of the ultrasonic transducer may be lead zirconate titanate (PZT) or lithium niobate. The acoustophoretic device may further comprise a cooling unit for cooling the ultrasonic transducer.

[0011] Also disclosed herein are methods for causing transduction of cells. The cells and a viral vector comprising nucleic acids are placed in an acoustophoretic device comprising: an acoustic chamber; and an ultrasonic transducer including a piezoelectric material that can be driven to create a multi-dimensional acoustic standing wave, a planar acoustic standing wave, or a combination of planar and multidimensional acoustic standing waves in the acoustic chamber. The ultrasonic transducer is driven to create the multi-dimensional acoustic standing wave, planar acoustic standing wave, or combination of multidimensional and planar acoustic standing waves. The cells and the viral vector are co-located by the acoustic standing wave to cause transduction of the cells.

[0012] Also disclosed are methods for causing transfection of cells. Pores are opened in the cell membranes of the cells. The cells are placed along with the nucleic acids in an acoustophoretic device comprising: an acoustic chamber; and an ultrasonic transducer including a piezoelectric material that can be driven to create an acoustic standing wave in the acoustic chamber. The acoustic standing wave can be a multi-dimensional acoustic standing wave, a planar acoustic standing wave, or a combination of planar and multidimensional acoustic standing waves. The pores can be opened before or after the cells are placed in the acoustophoretic device. The ultrasonic transducer is then driven to create the acoustic standing wave. The cells and the nucleic acids are co-located by the acoustic standing wave to cause transfection of the cells.

[0013] These and other non-limiting characteristics are more particularly described below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The accompanying drawings, which are incorporated in and constitute a part of this specification, show certain aspects of the subject matter disclosed herein and, together with the description, help explain some of the principles associated with the disclosed implementations.

[0015] **FIG. 1** is a diagram illustrating a method / process of the present disclosure, in which the efficiency of viral transduction is enhanced. A cell culture is combined with a viral vector tagged with green fluorescent protein (GFP) and exposed to acoustic

processing, where the multi-dimensional acoustic standing wave brings the cells and viruses into close proximity with each other, enhancing reaction efficiency. After washing and overnight incubation, GFP is expressed in the cells, thus showing transduction has occurred.

[0016] **FIG. 2A** is an exploded perspective view of an example acoustophoretic device according to the present disclosure including a cooling unit for cooling the transducer. **FIG. 2B** is a perspective view of the assembled device of **FIG. 2A**.

[0017] **FIG. 3** is a perspective view of another acoustophoretic device that can be used to practice the methods / processes of the present disclosure. A disposable container, such as a plastic bag, contains fluid mixture with two particle types that are caused to interact with each other in a separate acoustophoretic device containing one or more ultrasonic transducers.

[0018] **FIG. 4** is a cross-sectional diagram of a conventional ultrasonic transducer.

[0019] **FIG. 5** is a cross-sectional diagram of an ultrasonic transducer of the present disclosure. An air gap is present within the transducer, and no backing layer or wear plate are present.

[0020] **FIG. 6** is a cross-sectional diagram of an ultrasonic transducer of the present disclosure. An air gap is present within the transducer, and a backing layer and wear plate are present.

[0021] **FIG. 7** is a graph of electrical impedance amplitude versus frequency for a square transducer driven at different frequencies.

[0022] **FIG. 8** illustrates the trapping line configurations for seven of the resonance frequencies (minima of electrical impedance amplitudes) of **FIG. 7** from the direction orthogonal to fluid flow.

[0023] **FIG. 9** is a computer simulation of the acoustic pressure amplitude (right-hand scale in Pa) and transducer out of plane displacement (left-hand scale in meters). The text at the top of the left-hand scale reads "$x10^{-7}$". The text at the top of the left-hand scale by the upward-pointing triangle reads "$1.473x10^{-6}$". The text at the bottom of the left-hand scale by the downward-pointing triangle reads "$1.4612x10^{-10}$". The text at the top of the right-hand scale reads "$x10^{6}$". The text at the top of the right-hand scale by the upward-pointing triangle reads "$1.1129x10^{6}$". The text at the bottom of the right-hand scale by the

downward-pointing triangle reads “7.357”. The triangles show the maximum and minimum values depicted in this figure for the given scale. The horizontal axis is the location within the chamber along the X-axis, in inches, and the vertical axis is the location within the chamber along the Y-axis, in inches.

[0024] **FIG. 10** shows the In-Plane and Out-of-Plane displacement of a crystal where composite waves are present.

[0025] **FIG. 11** is a picture of a plastic bag in which T-cells and viruses are interacting with each other.

[0026] **FIG. 12** is a graph showing the ratio of transduction efficiency (acoustics / no acoustics) versus transduction time for three experiments with different run times (30, 60, and 90 minutes).

DETAILED DESCRIPTION

[0027] The present disclosure may be understood more readily by reference to the following detailed description of desired embodiments and the examples included therein. In the following specification and the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings.

[0028] Although specific terms are used in the following description for the sake of clarity, these terms are intended to refer only to the particular structure of the embodiments selected for illustration in the drawings, and are not intended to define or limit the scope of the disclosure. In the drawings and the following description below, it is to be understood that like numeric designations refer to components of like function.

[0029] The singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

[0030] The term "comprising" is used herein as requiring the presence of the named component and allowing the presence of other components. The term "comprising" should be construed to include the term "consisting of", which allows the presence of only the named component, along with any impurities that might result from the manufacture of the named component.

[0031] Numerical values should be understood to include numerical values which are the same when reduced to the same number of significant figures and numerical values

which differ from the stated value by less than the experimental error of conventional measurement technique of the type described in the present application to determine the value.

[0032] All ranges disclosed herein are inclusive of the recited endpoint and independently combinable (for example, the range of "from 2 grams to 10 grams" is inclusive of the endpoints, 2 grams and 10 grams, and all the intermediate values). The endpoints of the ranges and any values disclosed herein are not limited to the precise range or value; they are sufficiently imprecise to include values approximating these ranges and/or values.

[0033] The modifier "about" used in connection with a quantity is inclusive of the stated value and has the meaning dictated by the context. When used in the context of a range, the modifier "about" should also be considered as disclosing the range defined by the absolute values of the two endpoints. For example, the range of "from about 2 to about 10" also discloses the range "from 2 to 10." The term "about" may refer to plus or minus 10% of the indicated number. For example, "about 10%" may indicate a range of 9% to 11 %, and "about 1" may mean from 0.9-1.1.

[0034] It should be noted that many of the terms used herein are relative terms. For example, the terms "upper" and "lower" are relative to each other in location, i.e. an upper component is located at a higher elevation than a lower component in a given orientation, but these terms can change if the device is flipped. The terms "inlet" and "outlet" are relative to a fluid flowing through them with respect to a given structure, e.g. a fluid flows through the inlet into the structure and flows through the outlet out of the structure. The terms "upstream" and "downstream" are relative to the direction in which a fluid flows through various components, i.e. the flow fluids through an upstream component prior to flowing through the downstream component. It should be noted that in a loop, a first component can be described as being both upstream of and downstream of a second component.

[0035] The terms "horizontal" and "vertical" are used to indicate direction relative to an absolute reference, i.e. ground level. However, these terms should not be construed to require structures to be absolutely parallel or absolutely perpendicular to each other. For example, a first vertical structure and a second vertical structure are not necessarily

parallel to each other. The terms "top" and "bottom" or "base" are used to refer to surfaces where the top is always higher than the bottom/base relative to an absolute reference, i.e. the surface of the earth. The terms "upwards" and "downwards" are also relative to an absolute reference; upwards is always against the gravity of the earth.

[0036] The present application refers to "the same order of magnitude." Two numbers are of the same order of magnitude if the quotient of the larger number divided by the smaller number is a value of at least 1 and less than 10.

[0037] The acoustophoretic devices discussed herein may operate in a multimode or planar mode or a combination of both. Multimode refers to generation of acoustic waves by an acoustic transducer that create acoustic forces in three dimensions. The multimode acoustic waves, which may be ultrasonic, can be generated by a single acoustic transducer, and are sometimes referred to herein as multi-dimensional or three-dimensional acoustic standing waves. Planar mode refers to generation of acoustic waves by an acoustic transducer that create acoustic forces substantially in one dimension, e.g. along the direction of propagation. Such acoustic waves, which may be ultrasonic, that are generated in planar mode are sometimes referred to herein as one-dimensional acoustic standing waves.

[0038] The acoustic transducers may comprise a piezoelectric material, such as lead zirconate titanate (PZT) or lithium niobate. Such acoustic transducers can be electrically excited to generate planar or multimode acoustic waves. The three-dimensional acoustic forces generated by multimode acoustic waves include radial or lateral forces that are unaligned with a direction of acoustic wave propagation. The lateral forces may act in two dimensions. The lateral forces are in addition to the axial forces in multimode acoustic waves, which are substantially aligned with the direction of acoustic wave propagation. The lateral forces can be of the same order of magnitude as the axial forces for such multimode acoustic waves. The acoustic transducer excited in multimode operation may exhibit a standing wave on its surface, thereby generating a multimode acoustic wave. The standing wave on the surface of the transducer may be related to the mode of operation of the multimode acoustic wave. When an acoustic transducer is electrically excited to generate planar acoustic waves, the surface of the transducer may exhibit a piston-like action, thereby generating a one-dimensional acoustic standing wave.

Compared to planar acoustic waves, multimode acoustic waves exhibit significantly greater particle trapping activity on a continuous basis with the same input power. One or more acoustic transducers may be used to generate combinations of planar and multi-dimensional acoustic standing waves. For example, two acoustic transducers may be arranged to oppose each other to generate a standing wave. In such an example, one of the acoustic transducers may be passive and act as a reflector of an incident wave. Alternately, or in addition, each transducer may be active to generate acoustic waves, including such acoustic waves as described elsewhere herein.

[0039] Acoustophoresis is the manipulation of materials using acoustic waves. In some example implementations, acoustophoresis is used for separation of materials, and may represent a low-power, no-pressure-drop, no-clog, solid-state approach to particle separation from fluid dispersions. The scattering of the acoustic field off the particles results in a three-dimensional acoustic radiation force, which acts as a three-dimensional trapping field. The acoustic radiation force is proportional to the particle volume (e.g., the cube of the radius) when the particle is small relative to the wavelength. The acoustic radiation force is proportional to frequency and the acoustic contrast factor. The acoustic radiation force scales with acoustic energy (e.g., the square of the acoustic pressure amplitude). For harmonic excitation, the sinusoidal spatial variation of the force is what drives the particles to the stable positions within the standing waves. When the acoustic radiation force exerted on the particles is stronger than the combined effect of fluid drag force and buoyancy/gravitational force, the particle is trapped within the acoustic standing wave field. The action of the lateral and axial acoustic forces on the trapped particles results in formation of tightly packed clusters through concentration, clustering, clumping, agglomeration and/or coalescence of particles that, when reaching a critical size, settle continuously through enhanced gravity for particles heavier than the host fluid or rise out through enhanced buoyancy for particles lighter than the host fluid. Additionally, secondary inter-particle forces, such as Bjerkness forces, aid in particle agglomeration.

[0040] The acoustic standing waves create localized regions of high and low pressure. Particles are pushed to the standing wave nodes or antinodes depending on their compressibility and density relative to the surrounding fluid. Particles of higher density and compressibility move to the nodes in the standing waves, while secondary phases of

lower density move to the antinodes. The force exerted on the particles also depends on their size, with larger particles experiencing larger forces. The magnitude of the force depends on the particle density and compressibility relative to the fluid medium, and increases with the particle volume.

[0041] For purposes of the present disclosure, biological cells can be considered as particles. Most biological cell types present a higher density and lower compressibility than the medium in which they are suspended, so that the acoustic contrast factor between the cells and the medium has a positive value. As a result, the axial acoustic radiation force (ARF) drives the cells towards the standing wave pressure nodes. The axial component of the acoustic radiation force drives the cells, with a positive contrast factor, to the pressure nodes, whereas cells or other particles with a negative contrast factor are driven to the pressure anti-nodes. The radial or lateral component of the acoustic radiation force is the force that traps the cells. The radial or lateral component of the ARF is larger than the combined effect of fluid drag force and gravitational force.

[0042] Additional theoretical and numerical models have been developed for the calculation of the acoustic radiation force for a particle without any restriction as to particle size relative to wavelength. These models also include the effect of fluid and particle viscosity, and therefore are a more accurate calculation of the acoustic radiation force. The models that were implemented are based on the theoretical work of Yurii Ilinskii and Evgenia Zabolotskaya as described in AIP Conference Proceedings, Vol. 1474-1, pp. 255-258 (2012). Additional in-house models have been developed to calculate acoustic trapping forces for cylindrical shaped objects, such as the "hockey pucks" of trapped particles in the standing wave, which closely resemble a cylinder.

[0043] Desirably, the ultrasonic transducer(s) generates a multi-dimensional standing wave in the fluid that exerts a lateral force on the suspended particles to accompany the axial force. Typical results published in literature state that the lateral force is two orders of magnitude smaller than the axial force. In contrast, the technology disclosed in this application provides for a lateral force to be of the same order of magnitude as the axial force. However, in certain embodiments described further herein, the device use both transducers that produce multi-dimensional acoustic standing waves and transducers that produce planar acoustic standing waves. The lateral force component of the total

acoustic radiation force (ARF) generated by the ultrasonic transducer(s) of the present disclosure is significant and is sufficient to overcome the fluid drag force at linear velocities of up to 1 cm/s, and to create tightly packed clusters, and is of the same order of magnitude as the axial force component of the total acoustic radiation force.

[0044] The present disclosure relates to methods of using such acoustophoretic devices containing ultrasonic transducers to perform transfection or transduction. Briefly, the acoustophoretic devices are used to bring cells together with nucleic acids (either DNA or RNA, or both) in a localized volume. This permits the nucleic acids to be transferred into the cells. Current transduction processes can have relatively high cost, low efficiency, and poor ability to be scaled up for commercialization. The methods described herein can reduce cost, increase efficiency, and have a scalable platform for commercialization.

[0045] Very generally, the cells and the carrier (containing RNA or DNA) are placed in the acoustic chamber of an acoustophoretic device. Generally, they are suspended in a fluid to form a fluid mixture. In some example implementations, the acoustophoretic device contains an acoustic chamber that has an ultrasonic transducer and a reflector opposite the ultrasonic transducer (e.g. on opposite walls of the chamber). The ultrasonic transducer includes a piezoelectric material that can be driven to create an acoustic standing wave within the acoustic chamber, for example a multi-dimensional acoustic standing wave and/or a planar acoustic standing wave. The acoustic standing wave has locales of higher pressure and locales of lower pressure. In some examples, the lower pressure locales trap and retain the cells. The nucleic acids are flowed into the acoustic chamber with the retained cells. This action causes the cells and the nucleic acids to be co-located so the nucleic acids can enter the cells. In the case of transfection, pores are opened in the cell membranes of the cells, to permit naked nucleic acids to enter the cells. In the case of transduction, the nucleic acids are part of a viral vector. The viral vector enters the cells or inserts the nucleic acids into the cells. Specific viral vectors that may be used for performing transduction in the present disclosure include adenovirus, lentivirus, or paramyxovirus.

[0046] In some example implementations, the acoustophoretic force created by the acoustic standing wave on the cells and/or the nucleic acids (naked or in a viral vector)

can be sufficient to overcome the fluid drag force exerted by the moving fluid on these particles. In other words, the acoustophoretic force can act as a mechanism that traps the cells and/or the nucleic acids in the acoustic field. The acoustophoretic force can drive the cells and/or the nucleic acids to the stable locations of reduced or minimum acoustophoretic force amplitudes. These locations of reduced or minimum acoustophoretic force amplitudes can be the nodes of a standing acoustic wave. Over time, the collection of cells and/or nucleic acids at the nodes grows steadily. Within some period of time, which can be minutes or less depending on their concentration, the collection of cells and/or nucleic acids can assume the shape of a beam-like collection of disks. Each disk can be spaced by a half wavelength of the acoustic field.

[0047] In some embodiments, the acoustic standing wave traps the cells and/or the nucleic acids and co-locates them, improving the efficiency of the transfection / transduction reaction. A number of different mechanisms may be implemented for these embodiments. In one such mechanism, the cells and the nucleic acids may have similar acoustic contrast factors, such that both types of particles can be driven to the nodes or anti-nodes of the standing wave. This mechanism brings the cells and the nucleic acids in close spatial proximity with each other more efficiently than reliance on Brownian motion (as with conventional stirring). Put another way, the cells and the nucleic acids are trapped in a small three-dimensional volume created by the multi-dimensional acoustic standing wave, relative to the size of the acoustic chamber. In particular example embodiments, the cells and the nucleic acids either both have a positive acoustic contrast factor, or both have a negative acoustic contrast factor. Put another way, their acoustic contrast factors have the same sign. Again, the nucleic acids can be naked or in a viral vector.

[0048] In another mechanism, one of the two types of particles (cells or nucleic acids) may be driven to the nodes, while the other type of particles is driven to the anti-nodes. However, at higher frequencies, the nodes and anti-nodes are sufficiently close to each other that the cells and the nucleic acids can react with each other. In such embodiments, the cells or the nucleic acids have a positive acoustic contrast factor, and the other set (i.e. nucleic acids or the cells) has a negative acoustic contrast factor. Put another way, their acoustic contrast factors have opposite signs. The particles with a positive contrast

factor are driven to the nodes, and the particles with a negative contrast factor are driven to the anti-nodes. Some relevant factors for this reaction mechanism include the sizes of the cells and the carrier, and the frequency at which the ultrasonic transducer is operated.

[0049] Eventually, as the cells and/or the nucleic acids continue to be captured and concentrated, they can attain a size and weight such that gravitational settling will occur, wherein the clusters of particles will fall out of the acoustic standing wave to the bottom of the acoustic chamber. New collections of particles can then be trapped and reacted within the acoustic field generated by the acoustic standing waves.

[0050] In some example implementations, an acoustophoretic device is provided with an acoustic chamber that includes an ultrasonic transducer that includes a piezoelectric material that can be driven to create an acoustic standing wave within the acoustic chamber, for example a multi-dimensional acoustic standing wave and/or a planar acoustic standing wave. The acoustic standing wave has locales of higher pressure and locales of lower pressure. A recirculation loop is provided to the acoustic chamber that permits output fluid and particles to be reintroduced to the acoustic chamber. In some examples, the lower pressure locales trap and retain the cells, but do not trap or retain the viral vector or nucleic acids, which can flow out of the acoustic chamber and be reintroduced via the recirculation loop. In the example of viral vectors, the cells are retained in the acoustic field generated by the acoustic standing wave, and the viral vectors are flowed into the field to permit the cells and viral vectors to interact. Increased numbers of interactions can be attained between the viral vectors and the cells by recirculating to the acoustic chamber the viral vectors that do not interact on a prior pass. In the example of nucleic acids, an additional treatment can be performed on the cells that are retained in the acoustic field to permit pores to be formed in the cell membranes, as discussed in greater detail elsewhere herein. The nucleic acids are flowed into the acoustic chamber with the retained cells to permit the nucleic acids to interact with the cells and to permit the nucleic acids to enter the cells via the pores. The nucleic acids are not retained by the acoustic field, and are recirculated to the acoustic chamber to permit multiple passes at transfection to occur.

[0051] In addition, or alternatively, in some examples, the ultrasonic transducer is driven to cause acoustic streaming within the acoustic chamber. Briefly, acoustic

streaming refers to the fluid flow that results within the acoustic chamber when the fluid absorbs the acoustic energy that is transmitted by the ultrasonic transducer (from the vibration of the ultrasonic transducer). The velocity of the fluid is induced by the oscillating acoustic waves generated by the ultrasonic transducer. Typically, when acoustic streaming is generated, it results in circulatory motion or vortices that can cause stirring in the fluid mixture. This phenomenon is nonlinear, and can cause the cells and the nucleic acids to interact with each other.

[0052] The cells and the nucleic acids are brought into proximity so that they can react with each other. In the present disclosure, the terms “interact” and “react” are used to indicate that a physical change occurs in the cells. For example, in transduction, the virus containing the nucleic acids may penetrate into the cell to cause transduction to occur. The virus can be, for example, a retrovirus such as a lentivirus.

[0053] With regard to transfection examples, pores are opened in the cell membranes of the cells to permit nucleic acids to enter the cells. For example, the cells could be subjected to electroporation, where the cells are exposed to an electric field to increase the permeability of the cell membrane. Sonoporation could also be used, where the cells are exposed to ultrasound to induce pore formation in the cell membrane. Calcium phosphate could also be used to cause transfection. The location at which the pore opening occurs is not important, and can occur in the acoustophoretic device upstream of the acoustic chamber in which the cells and the nucleic acids are mixed, or can occur outside of the acoustophoretic device.

[0054] For reference, it is noted that productive transfection and gene transfer require not simply the entry of DNA into cells and subsequent transcription from an appropriate promoter, but also a number of intracellular events that allow the DNA to move from the extracellular surface of the cell into and through the cytoplasm, and ultimately across the nuclear envelope and into the nucleus before any transcription can initiate. Immediately upon entry into the cytoplasm, naked DNA (either delivered through physical techniques or after disassembly of DNA–carrier complexes) associates with a large number of cellular proteins that mediate subsequent interactions with the microtubule network for movement toward the microtubule organizing center and the nuclear envelope. Plasmids then enter the nucleus either upon the mitotic disassembly of the nuclear envelope or

through nuclear pore complexes in the absence of cell division, using a different set of proteins.

[0055] Specific viral vectors that may be used for performing transduction in the present disclosure include adenovirus, lentivirus, or paramyxovirus. Retroviruses are characterized by their ability to retrotranscribe their RNA genome into a cDNA copy, which is then stably integrated into the host cell genome. Thus, the virus carries the nucleic acid into the cell.

[0056] Retroviruses can be categorized as simple or complex (e.g., lentiviruses). The The viral particles of both types contain two copies of positive-stranded RNA with an associated viral reverse transcriptase (RT) located within an internal core. Also located within this compartment are structural and enzymatic proteins, including the nucleocapsid (NC), capsid (CA), integrase (IN), and protease (PR). The inner core is surrounded by an outer protein layer comprised of the matrix (MA) protein, which is in turn encompassed by the envelope glycoprotein (ENV)-studded, host cell membrane-derived envelope.

[0057] Lentiviral and retroviral gene delivery systems (or carriers) exploit aspects of retrovirus replication to provide stable integration of the desired nucleic acid sequence. Whereas transfection of foreign nucleic acids results only in transient transgene expression, the activity of the viral integrase in retroviral and lentiviral-based systems allows for stable integration of the foreign transgene, which is then inherited and continuously expressed over repeated cell divisions. A key feature of both lentiviral and retroviral vectors is that they produce replication-defective, or self-inactivating, particles. This allows for delivery of the desired sequence, without continued viral replication in the target cells.

[0058] A common way to validate that the foreign nucleic acids were successfully introduced into cells is to measure protein expression. This is typically performed by Western blot or immunostaining.

[0059] Examples of cells that can be transfected / transducted by the processes of the present disclosure include Chinese hamster ovary (CHO) cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, human cells, regulatory T-cells, helper T-cells, cytotoxic T-cells, memory T-cells, effector T-cells, gamma delta T-cells, Jurkat T-cells, CAR-T cells, B cells, or NK cells, peripheral blood mononuclear cells (PBMCs), algae, plant cells, or

bacteria. The cells may themselves be attached to other materials, such as beads. Examples of beads include polymer beads, magnetic beads, superparamagnetic beads, and microspheres. These can be used for biochemical reactions or for labeling purposes.

[0060] In some embodiments, additives can be included in the mixture of the cells and the nucleic acids. Such additives can include Polybrene™ and RetroNectin™. Polybrene™ is a cationic polymer (polyhexadimethrine bromide) used to increase the efficiency of transduction of certain cells with retrovirus (and lentivirus) in cell culture, and is typically used in amounts of 10 µg/mL or less. RetroNectin™ is a polypeptide consisting of three functional domains derived from human fibronectin protein (C-domain, H-doman, and CS-1 site). RetroNectin™ enhances the efficiency of retroviral- and lentiviral-mediated transduction in hematopoietic cells including hematopoietic stem cells and terminally differentiated cells such as primary T cells and macrophages. Other additives can include lipofectin, lipofectamine, and cationic peptides such as protamine. These additives can be used to enhance transduction if appropriate.

[0061] Without being limited by theory, it is believed that the frequency of the multi-dimensional acoustic standing wave determines the diameter of the particles that can be trapped by the acoustic standing wave. For example, for a 2 MHz wave, the particle size is about 1 to about 100 microns.

[0062] **FIG. 1** is a diagram illustrating an example method of the present disclosure, as applied to viral transduction. In this example, cells are labeled with green fluorescent protein (GFP). Starting from the left-hand side of the figure, first, a cell culture **100** is combined with a viral vector **110**. The fluid mixture containing the cells and the viruses are then placed in an acoustic chamber **120**, which is located between an ultrasonic transducer **122** and a reflector **124**. Acoustic standing waves are generated for 10 minutes at room temperature. As illustrated here, the cells and the viruses are trapped in the acoustic standing waves. The cells are trapped at the nodes and the viruses are trapped at the anti-nodes. However, due to their relative size, the cells and viruses are co-located, and the viruses are able to infect the cells (identified with reference numeral **128**). After washing to remove unreacted material, the cells are incubated overnight at 37°C and GFP is expressed in labeled cells. A similar method can be used to make T-cells that express chimeric antigen receptors (CARs), or CAR T-cells.

[0063] The methods of the present disclosure can be carried out in a continuous process, wherein a fluid mixture containing the cells and the nucleic acids suspended in a host fluid is flowed through the acoustophoretic device. The nucleic acids can be naked, or they can contained in a viral vector, such as a bacteriophage or other virus.

[0064] **FIG. 2A** is an exploded view of an acoustophoretic device **200** that can be used for continuous processing. **FIG. 2B** is a view of the device **200** in a fully assembled condition.

[0065] Referring to **FIG. 2A**, the acoustophoretic device can be built such that each component is modular, and can be changed or switched out separately from each other. Thus, when new revisions or modifications are made to a given component, the component can be replaced while the remainder of the device stays the same.

[0066] The device includes an ultrasonic transducer **220** and a reflector **250** on opposite walls of an acoustic chamber **210**. It is noted that the reflector **250** may be made of a transparent material, such that the interior of the flow chamber **210** can be seen. The ultrasonic transducer is proximate a first wall of the acoustic chamber. The reflector is proximate a second wall of the acoustic chamber or can make up the second wall of the acoustic chamber.

[0067] A cooling unit **260** can be located between the ultrasonic transducer **220** and the flow chamber **210**. As illustrated here, the cooling unit **260** includes an independent flow path that is separate from the flow path through the acoustic chamber. A coolant inlet **262** permits the ingress of a cooling fluid into the cooling unit. The coolant and waste heat exit the cooling unit through a coolant outlet **264**. The coolant that flows through the cooling unit can be any appropriate fluid. For example, the coolant can be water, air, alcohol, ethanol, ammonia, or some combination thereof. The coolant can be a liquid, gas, or gel. The coolant can be an electrically non-conductive fluid to prevent electric short-circuits.

[0068] Alternatively, the cooling unit can be in the form of a thermoelectric generator, which converts heat flux (i.e. temperature differences) into electrical energy using the Seebeck effect, thus removing heat from the flow chamber. Put another way, electricity can be generated from undesired waste heat while operating the acoustophoretic device.

[0069] The cooling unit can be used to cool the ultrasonic transducer, which can be particularly advantageous when the device is to be run continuously with repeated processing and recirculation for an extended period of time (e.g., perfusion). Alternatively, the cooling unit can also be used to cool the fluid running through the acoustic chamber **210**. For desired applications, the cells should be maintained around room temperature (~20°C), and at most around 28°C. This is because when cells experience higher temperatures, their metabolic rates increase. Without a cooling unit, however, the temperature of the cells flowing through the acoustic chamber can rise as high as 34°C.

[0070] It is noted that the acoustic chamber **210** is illustrated here as including at least an inlet **212** and an outlet **214**. This provides access to the interior volume **216** of the acoustic chamber. Additional inlets and outlets (e.g. fluid inlet, concentrate outlet, permeate outlet, recirculation outlet, bleed / harvest outlet) may be included as desired. The interior volume **216** can be considered as being bounded by the ultrasonic transducer **220**, the cooling unit **260**, the acoustic chamber **210**, and the reflector **250**.

[0071] The flow direction of the acoustophoretic device **200** can be oriented in a direction other than horizontal. For example, the fluid flow can be vertical either upward or downward or at some angle relative to vertical or horizontal. More than one transducer can be included in the system.

[0072] **FIG. 3** illustrates another acoustophoretic device **300** which can be used to practice the methods and processes of the present disclosure. Very generally, the system includes the acoustophoretic device **300** and a substantially acoustically transparent container **310**. These two components are separable from each other.

[0073] The container **310** of the acoustophoretic device is generally formed from a substantially acoustically transparent material such as plastic, glass, polycarbonate, low-density polyethylene, and high-density polyethylene (all at an appropriate thickness). However, the container may be formed from any material suitable for allowing the passage of the acoustic standing wave(s) of the present disclosure therethrough. The container may be in the form of a bottle or a bag. The difference between these forms lies in their composition and structure. A bottle is more rigid than a bag. When empty, a bag is generally unable to support itself, while a bottle is able to stand upright. For

example, the container **310** shown here is a high-density polyethylene bag. Container **310** generally has an upper end **312** and a lower end **314**, and an interior volume in which the fluid mixture (containing the first particles and second particles in a host fluid) is located.

[0074] The acoustophoretic device **300** is defined by at least one wall **332**, and usually a plurality of walls, which form its sides. For example, the acoustophoretic device may be in the shape of a cylinder, or in a rectangle (as depicted). The wall(s) are solid. An opening **326** is present in an upper end of the acoustophoretic device, for receiving the container **310** therethrough. Again, the acoustophoretic device **300** is separable from the container **310**, so that the container can be either disposable or reusable, depending upon the desired application of the acoustophoretic device. As illustrated here, the base of the acoustophoretic device **300** is solid.

[0075] The acoustophoretic device **300** includes at least one ultrasonic transducer **330** on a wall **334**. The ultrasonic transducer **330** has a piezoelectric material driven by a voltage signal to create an acoustic standing wave. Cables **332** are illustrated for transmitting power and control information to the ultrasonic transducer **330**. A reflector **340** may be present, and is located on the wall **336** opposite the ultrasonic transducer **330**. The standing wave is thus generated through initial waves radiated from the transducer and reflected waves from the reflector. In some embodiments, a separate reflector is not necessary. For example, a chamber wall or release boundary, such as may be provided by ambient air, may be used to reflect the incident waves and create the standing waves. It is to be understood that various transducer and reflector combinations may be used. The planar and/or multi-dimensional acoustic standing wave(s) are generated within the container, and are used to cause interaction of the particles within the container **310**. It should be noted that there is no contact between the ultrasonic transducer and the fluid mixture within the container **310**.

[0076] In certain embodiments, the acoustophoretic device includes a plurality of ultrasonic transducers **330** located on a common wall **334** opposite the wall **336** on which the reflector **340** is located. Alternatively, the ultrasonic transducers can be located opposite each other, with no reflector being present. Additionally, the acoustophoretic device **300** may include a viewing window **324** in another wall **338**. As illustrated here,

when a viewing window is provided, it can be in a wall adjacent the walls upon which the ultrasonic transducer(s) and reflector are located, such that the lower end **314** of the container **310** can be viewed through the viewing window **324** in the separation chamber **320**. In other embodiments, the viewing window can take the place of the reflector.

[0077] In certain embodiments, a fluid, such as water, may be placed in the interstitial space **305** between the container **310** and the acoustophoretic device **300**, such that the acoustic standing wave passes through both the fluid in the interstitial space and the fluid mixture in the container. The interstitial fluid can be any fluid, though it should have an acoustic impedance value that allows for good transmission of the acoustic standing wave(s), and preferably a low acoustic attenuation.

[0078] In particular example embodiments, the ultrasonic transducer is driven at a frequency of about 0.5 MHz to about 20 MHz (megahertz). Higher frequency standing wave fields result in steeper pressure gradients, which in turn are better suited for trapping smaller particles like viruses. The ultrasonic transducer can be driven for a time period of about 5 minutes to about 15 minutes. This is a considerably shorter time period than, for example, conventional viral transduction processes where the cell culture and viral vector are incubated together for about 30 minutes to about 120 minutes. Such lengthy incubation periods are due to the reaction between cells and viruses only occurring when Brownian motion brings them in proximity to each other. Using the acoustophoretic devices of the present disclosure greatly increases the probability of cells and viruses being in sufficient proximity to react with each other. This results in higher reaction efficiency using fewer particles. If desired, though, the ultrasonic transducer can be driven for as long a time period as desired, for example up to 120 minutes or longer.

[0079] It may be helpful now to describe the ultrasonic transducer(s) used in the acoustic filtering device in more detail. **FIG. 4** is a cross-sectional diagram of a conventional ultrasonic transducer. This transducer has a wear plate **50** at a bottom end, epoxy layer **52**, ceramic piezoelectric element **54** (made of, e.g. Lead Zirconate Titanate (PZT) or lithium niobate), an epoxy layer **56**, and a backing layer **58**. On either side of the ceramic piezoelectric element, there is an electrode: a positive electrode **61** and a negative electrode **63**. The epoxy layer **56** attaches backing layer **58** to the piezoelectric element **54**. The entire assembly is contained in a housing **60** which may be made out

of, for example, aluminum. The housing is used as the ground electrode. An electrical adapter **62** provides connection for wires to pass through the housing and connect to leads (not shown) which attach to the piezoelectric element **54**. Typically, backing layers are designed to add damping and to create a broadband transducer with uniform displacement across a wide range of frequency and are designed to suppress excitation of particular vibrational eigen-modes of the piezoelectric element. Wear plates are usually designed as impedance transformers to better match the characteristic impedance of the medium into which the transducer radiates.

[0080] **FIG. 5** is a cross-sectional view of an ultrasonic transducer **81** of the present disclosure, which is used in the acoustic filtering device of the present disclosure. Transducer **81** is shaped as a square, and has an aluminum housing **82**. The aluminum housing has a top end and a bottom end. The transducer housing may also be composed of plastics, such as medical grade HDPE or other metals. The piezoelectric element is a mass of perovskite ceramic, each consisting of a small, tetravalent metal ion, usually titanium or zirconium, in a lattice of larger, divalent metal ions, usually lead or barium, and O^{2-} ions. As an example, a PZT (lead zirconate titanate) piezoelectric element **86** defines the bottom end of the transducer, and is exposed from the exterior of the bottom end of the housing. The piezoelectric element is supported on its perimeter by a small elastic layer **98**, e.g. epoxy, silicone or similar material, located between the piezoelectric element and the housing. Put another way, no wear plate or backing material is present. However, in some embodiments, there is a layer of plastic or other material separating the piezoelectric element from the fluid in which the acoustic standing wave is being generated. The piezoelectric material / element / crystal has an exterior surface (which is exposed) and an interior surface as well.

[0081] Screws **88** attach an aluminum top plate **82a** of the housing to the body **82b** of the housing via threads. The top plate includes a connector **84** for powering the transducer. The top surface of the piezoelectric element **86** is connected to a positive electrode **90** and a negative electrode **92**, which are separated by an insulating material **94**. The electrodes can be made from any conductive material, such as silver or nickel. Electrical power is provided to the piezoelectric element **86** through the electrodes on the piezoelectric element. Note that the piezoelectric element **86** has no backing layer or

epoxy layer. Put another way, there is an interior volume or an air gap **87** in the transducer between aluminum top plate **82a** and the piezoelectric element **86** (i.e. the air gap is completely empty). A minimal backing **58** and/or wear plate **50** may be provided in some embodiments, as seen in **FIG. 6**.

[0082] The transducer design can affect performance of the system. A typical transducer is a layered structure with the ceramic piezoelectric element bonded to a backing layer and a wear plate. Because the transducer is loaded with the high mechanical impedance presented by the standing wave, the traditional design guidelines for wear plates, e.g., half wavelength thickness for standing wave applications or quarter wavelength thickness for radiation applications, and manufacturing methods may not be appropriate. Rather, in one embodiment of the present disclosure the transducers, there is no wear plate or backing, allowing the piezoelectric element to vibrate in one of its eigenmodes with a high Q-factor, or in a combination of several eigenmodes. The vibrating ceramic piezoelectric element/disk is directly exposed to the fluid flowing through the fluid cell.

[0083] Removing the backing (e.g. making the piezoelectric element air backed) also permits the ceramic piezoelectric element to vibrate at higher order modes of vibration with little damping (e.g. higher order modal displacement). In a transducer having a piezoelectric element with a backing, the piezoelectric element vibrates with a more uniform displacement, like a piston. Removing the backing allows the piezoelectric element to vibrate in a non-uniform displacement mode. The higher order the mode shape of the piezoelectric element, the more nodal lines the piezoelectric element has. The higher order modal displacement of the piezoelectric element creates more trapping lines, although the correlation of trapping line to node is not necessarily one to one, and driving the piezoelectric element at a higher frequency will not necessarily produce more trapping lines.

[0084] In some embodiments of the acoustic filtering device of the present disclosure, the piezoelectric element may have a backing that minimally affects the Q-factor of the piezoelectric element (e.g. less than 5%). The backing may be made of a substantially acoustically transparent material such as balsa wood, foam, or cork which allows the piezoelectric element to vibrate in a higher order mode shape and maintains a high Q-

factor while still providing some mechanical support for the piezoelectric element. The backing layer may be a solid, or may be a lattice having holes through the layer, such that the lattice follows the nodes of the vibrating piezoelectric element in a particular higher order vibration mode, providing support at node locations while allowing the rest of the piezoelectric element to vibrate freely. The goal of the lattice work or acoustically transparent material is to provide support without lowering the Q-factor of the piezoelectric element or interfering with the excitation of a particular mode shape.

[0085] Placing the piezoelectric element in direct contact with the fluid also contributes to the high Q-factor by avoiding the dampening and energy absorption effects of the epoxy layer and the wear plate. Other embodiments of the transducer(s) may have wear plates or a wear surface to prevent the PZT, which contains lead, contacting the host fluid. This may be desirable in, for example, biological applications such as separating blood, biopharmaceutical perfusion, or fed-batch filtration of mammalian cells. Such applications might use a wear layer such as chrome, electrolytic nickel, or electroless nickel. Chemical vapor deposition could also be used to apply a layer of poly(p-xylylene) (e.g. Parylene) or other polymer. Organic and biocompatible coatings such as silicone or polyurethane are also usable as a wear surface. Thin films, such as a polyetheretherketone (PEEK) film, can also be used as a cover of the transducer surface exposed to the fluid with the advantage of being a biocompatible material. In one embodiment, the PEEK film is adhered to the face of the piezoelectric material using pressure sensitive adhesive (PSA). Other films can be used as well.

[0086] In some embodiments, the ultrasonic transducer has a nominal 2 MHz resonance frequency. Each transducer can consume about 28 W of power for droplet trapping at a flow rate of 3 GPM (gallons per minute). This translates to an energy cost of 0.25 kW hr/ m^3. This is an indication of the very low cost of energy of this technology. Desirably, each transducer is powered and controlled by its own amplifier. In other embodiments, the ultrasonic transducer uses a square piezoelectric element, for example with 1"x1" dimensions. Alternatively, the ultrasonic transducer can use a rectangular piezoelectric element, for example with 1"x2.5" dimensions. Power dissipation per transducer was 10 W per 1"x1" transducer cross-sectional area and per inch of acoustic standing wave span in order to get sufficient acoustic trapping forces. For a 4" span of an

intermediate scale system, each 1"x1" square transducer consumes 40 W. The larger 1"x2.5" rectangular transducer uses 100W in an intermediate scale system. The array of three 1"x1" square transducers would consume a total of 120W and the array of two 1"x2.5" transducers would consume about 200 W. Arrays of closely spaced transducers represent alternate potential embodiments of the technology. Transducer size, shape, number, and location can be varied as desired to generate desired multi-dimensional acoustic standing wave patterns.

[0087] The size, shape, and thickness of the transducer determine the transducer displacement at different frequencies of excitation, which in turn affects separation efficiency. Typically, the transducer is operated at frequencies near the thickness resonance frequency (half wavelength). Gradients in transducer displacement typically result in more trapping locations for the cells/biomolecules. Higher order modal displacements generate three-dimensional acoustic standing waves with strong gradients in the acoustic field in all directions, thereby creating equally strong acoustic radiation forces in all directions, leading to multiple trapping lines, where the number of trapping lines correlate with the particular mode shape of the transducer.

[0088] To investigate the effect of the transducer displacement profile on acoustic trapping force and separation efficiencies, an experiment was repeated ten times using a 1”x1” square transducer, with all conditions identical except for the excitation frequency. Ten consecutive acoustic resonance frequencies, indicated by circled numbers 1-9 and letter A on **FIG. 7**, were used as excitation frequencies. The conditions were experiment duration of 30 min, a 1000 ppm oil concentration of approximately 5-micron SAE-30 oil droplets, a flow rate of 500 ml/min, and an applied power of 20W. Oil droplets were used because oil is less dense than water, and can be separated from water using acoustophoresis.

[0089] **FIG. 7** shows the measured electrical impedance amplitude of a square transducer as a function of frequency in the vicinity of the 2.2 MHz transducer resonance. The minima in the transducer electrical impedance correspond to acoustic resonances of the water column and represent potential frequencies for operation. Additional resonances exist at other frequencies where multi-dimensional standing waves are excited. Numerical modeling has indicated that the transducer displacement profile varies

significantly at these acoustic resonance frequencies, and thereby directly affects the acoustic standing wave and resulting trapping force. Since the transducer operates near its thickness resonance, the displacements of the electrode surfaces are essentially out of phase. The typical displacement of the transducer electrodes is not uniform and varies depending on frequency of excitation. As an example, at one frequency of excitation with a single line of trapped oil droplets, the displacement has a single maximum in the middle of the electrode and minima near the transducer edges. At another excitation frequency, the transducer profile has multiple maxima leading to multiple trapped lines of oil droplets. Higher order transducer displacement patterns result in higher trapping forces and multiple stable trapping lines for the captured oil droplets.

[0090] As the oil-water emulsion passed by the transducer, the trapping lines of oil droplets were observed and characterized. The characterization involved the observation and pattern of the number of trapping lines across the fluid channel, as shown in **FIG. 8**, for seven of the ten resonance frequencies identified in **FIG. 7**. Different displacement profiles of the transducer can produce different (more) trapping lines in the standing waves, with more gradients in displacement profile generally creating higher trapping forces and more trapping lines.

[0091] **FIG. 9** is a numerical model showing a pressure field that matches the 9 trapping line pattern. The numerical model is a two-dimensional model; and therefore only three trapping lines are observed. Two more sets of three trapping lines exist in the third dimension perpendicular to the plane of the page.

[0092] The lateral force of the acoustic radiation force generated by the transducer can be increased by driving the transducer in higher order mode shapes, as opposed to a form of vibration where the crystal effectively moves as a piston having a uniform displacement. In some example implementations, the higher order mode shapes are Bessel functions. The acoustic pressure is proportional to the driving voltage of the transducer. The electrical power is proportional to the square of the voltage. The transducer is typically a thin piezoelectric plate, with electric field in the z-axis and primary displacement in the z-axis. The transducer is typically coupled on one side by air (i.e., the air gap within the transducer) and on the other side by the fluid mixture containing the particles that will be interacted with each other. The types of waves generated in the plate

are known as composite waves. A subset of composite waves in the piezoelectric plate is similar to leaky symmetric (also referred to as compressional or extensional) Lamb waves. The piezoelectric nature of the plate typically results in the excitation of symmetric Lamb waves. The waves are leaky because they radiate into the water layer, which result in the generation of the acoustic standing waves in the water layer. Lamb waves exist in thin plates of infinite extent with stress free conditions on its surfaces. Because the transducers of this embodiment are finite in nature, the actual modal displacements are more complicated.

[0093] **FIG. 10** shows the typical variation of the in-plane displacement (x-displacement) and out-of-plane displacement (y-displacement) across the thickness of the plate, the in-plane displacement being an even function across the thickness of the plate and the out-of-plane displacement being an odd function. Because of the finite size of the plate, the displacement components vary across the width and length of the plate. In general, a (m,n) mode is a displacement mode of the transducer in which there are m undulations in transducer displacement in the width direction and n undulations in the length direction, and with the thickness variation as described in **FIG. 10**. The maximum number of m and n is a function of the dimension of the piezoelectric material (e.g., a piezoelectric crystal) and the frequency of excitation. Additional three-dimensional modes exist that are not of the form (m,n).

[0094] The transducers are driven so that the piezoelectric element vibrates in higher order modes of the general formula (m, n), where m and n are independently 1 or greater. Generally, the transducers will vibrate in higher order modes than (2,2). Higher order modes will produce more nodes and antinodes, result in three-dimensional standing waves in the water layer, characterized by strong gradients in the acoustic field in all directions, not only in the direction of the standing waves, but also in the lateral directions. As a consequence, the acoustic gradients result in stronger trapping forces in the lateral direction.

[0095] Generally, the ultrasonic transducer(s) may be driven by an electrical signal, which may be controlled based on voltage, current, phase angle, power, frequency or any other electrical signal characteristic. In particular, the driving signal for the transducer may be based on voltage, current, magnetism, electromagnetism, capacitive or any other

type of signal to which the transducer is responsive. In embodiments, the voltage signal driving the transducer can have a sinusoidal, square, sawtooth, pulsed, or triangle waveform; and have a frequency of 500 kHz to 10 MHz. The voltage signal can be driven with pulse width modulation, which produces any desired waveform. The voltage signal can also have amplitude or frequency modulation start/stop capability to eliminate streaming. In particular embodiments, the voltage signal can have a frequency of about 0.5 MHz to about 30 MHz, so that such frequencies are produced by the ultrasonic transducer.

[0096] The transducers are used to create a pressure field that generates acoustic radiation forces of the same order of magnitude both orthogonal to the standing wave direction and in the standing wave direction. When the forces are roughly the same order of magnitude, particles of size 0.1 microns to 300 microns will be moved more effectively towards "trapping lines", so that the cells and the nucleic acids (whether naked or in a viral vector) are co-located next to each other, permitting them to react with each other.

[0097] In biological applications, all of the parts of the system (i.e., the bioreactor, acoustic filtering device, tubing fluidly connecting the same, etc.) can be separated from each other and be disposable. Avoiding centrifuges and filters allows better separation of the biological cells from fluid without lowering the viability of the cells. The transducers may also be driven to create rapid pressure changes to prevent or clear blockages due to agglomeration of biological cells. The frequency of the transducers may also be varied to obtain optimal effectiveness for a given power.

[0098] The techniques and implementations described herein may be used for integrated continuous automated bioprocessing. Control can be distributed to some or all units involved in the bioprocessing. Feedback from units can be provided to permit overview of the bioprocess, which may be in the form of screen displays, control feedbacks, reporting, status reports and other information conveyance. Distributed processing permits a high degree of flexibility in achieving a desired process control, for example by coordinating steps among units and providing a batch executive control.

[0099] The acoustophoretic devices utilizing an acoustic wave system can be implemented with biocompatible materials, and may include gamma sterilizable and single use components. The processing system also permits ultrasonic flow

measurement, which is noninvasive, and is capable of operating with high viscosity fluids. The system can be implemented with single use sterile septic connectors and a simple graphical user interface (GUI) for control. The acoustophoretic device is scalable. For example, a relatively small unit is capable of operation at 2 L to 50 L scale.

[0100] The methods, systems, and devices discussed above are examples. Various configurations may omit, substitute, or add various procedures or components as appropriate. For instance, in alternative configurations, the methods may be performed in an order different from that described, and that various steps may be added, omitted, or combined. Also, features described with respect to certain configurations may be combined in various other configurations. Different aspects and elements of the configurations may be combined in a similar manner. Also, technology evolves and, thus, many of the elements are examples and do not limit the scope of the disclosure or claims.

[0101] Specific details are given in the description to provide a thorough understanding of example configurations (including implementations). However, configurations may be practiced without these specific details. For example, well-known processes, structures, and techniques have been shown without unnecessary detail to avoid obscuring the configurations. This description provides example configurations only, and does not limit the scope, applicability, or configurations of the claims. Rather, the preceding description of the configurations provides a description for implementing described techniques. Various changes may be made in the function and arrangement of elements without departing from the spirit or scope of the disclosure.

[0102] A statement that a value exceeds (or is more than) a first threshold value is equivalent to a statement that the value meets or exceeds a second threshold value that is slightly greater than the first threshold value, e.g., the second threshold value being one value higher than the first threshold value in the resolution of a relevant system. A statement that a value is less than (or is within) a first threshold value is equivalent to a statement that the value is less than or equal to a second threshold value that is slightly lower than the first threshold value, e.g., the second threshold value being one value lower than the first threshold value in the resolution of the relevant system.

[0103] Also, configurations may be described as a process that is depicted as a flow diagram or block diagram. Although each may describe the operations as a sequential

process, many of the operations can be performed in parallel or concurrently. In addition, the order of the operations may be rearranged. A process may have additional stages or functions not included in the figure.

[0104] The following examples are provided to illustrate the devices and processes of the present disclosure. The examples are merely illustrative and are not intended to limit the disclosure to the materials, conditions, or process parameters set forth therein.

EXAMPLES

[0105] **Example 1:**

[0106] **FIG. 11** is a picture of a plastic bag containing a fluid mixture with T-cells and viruses. The plastic bag was placed into an acoustophoretic device that was filled with water. A multi-dimensional acoustic standing wave was generated, causing the T-cells and viruses to interact with each other. This is visible as a series of beams of disks within the plastic bag.

[0107] **Example 2:**

[0108] The BacMam® system (ThermoFisher Scientific) uses baculoviruses for transduction, and was used for transduction of green fluorescent protein (GFP) into Jurkat T-cells. This system was used for various experiments. Five results are shown below. They were labeled Control, Process Control 1, Process Control 2, Acoustics 3MHz, and Acoustics 10MHz.

[0109] The Control experiment, the Process Control 1 experiment, and the Process Control 2 experiment were not exposed to acoustic standing waves.

[0110] For the Acoustics 3MHz experiment, interaction between the T-cells and viruses was enhanced using an acoustic standing wave of nominal frequency 3 Hz.

[0111] For the Acoustics 10MHz experiment, interaction between the T-cells and viruses was enhanced using an acoustic standing wave of nominal frequency 10 Hz.

[0112] The results are listed in Table A below. The MOI is the multiplicity of infection, or the number of viral vector particles per cell. The GFP+ is the % of cells that expressed GFP.

Table A.

Experiment	MOI	GFP+ (%)
Control	-	-
Process Control 1	50	28.4
Process Control 2	50	48.8
Acoustics 3MHz	10	21.8
Acoustics 10MHz	10	48.4

[0113] Using acoustics resulted in equivalent transduction efficiency with 80% fewer viral particles per cell.

[0114] **Example 3:**

[0115] An acoustic chamber included an ultrasonic transducer and a reflector. A recirculation loop drew fluid from one end of the acoustic chamber and then circulated the fluid back through the other end into the acoustic chamber. The acoustic chamber had a volume of about 1 mL, and the recirculation loop had a volume of about 3 mL. Transduction tests were performed using the acoustic chamber.

[0116] Four acoustic tests were performed using the acoustic chamber with recirculation. For two Acoustic tests, the acoustics were turned on at a power level of 1 W, to determine the effect of acoustics on transduction efficiency. For the other two tests, the acoustics were turned off (Recirculation only).

[0117] Each Acoustic test was also performed with a Control run and a Static Run. For the Control run, virus was added to cells in a Petri dish, and then immediately washed out. For the Static run, virus was added to cells in a Petri dish for 90 minutes, then washed out. The MOI (the number of viral vector particles per cell) for these tests was 25. For all runs, cell viability was measured by nucleocounter. For each Acoustic run, cell viability was also measured using a Vi-Cell cell viability analyzer.

[0118] Table B indicates the parameters of each test. Table C indicates the results of each test run. Table D provides the Vi-Cell analysis. Table E provides the Nucleocounter analysis. In Table C, the transduction gain is the Acoustic efficiency divided by the Static efficiency.

Table B.

Test	Volume (mL)	Flow Rate (mL/min)	Acoustic Power (W)	Total Number of Cells (million)	Recirculations
1	4.13	4.13	1	8	90
2	4.13	4.13	1	8	90
3	4.13	4.13	N/A (recirc only)	8	90
4	4.13	4.13	N/A (recirc only)	8	90

Table C.

				Viability		
Test	Run	Efficiency (%)	Std. Dev. (%)	Nucleo counter (%)	Vi-Cell (%)	Transduction Gain
1	Control	1.6	0.2	96		
1	Static	17.5	0.7	97		
1	Acoustic	35.4	1.6	96	93	2.02
2	Control	1.3	0.1	96		
2	Static	18.7	1.7	94		
2	Acoustic	28.8	1.6	96	90	1.53
3	Control	2.9	0.2	96		
3	Static	14.9	1.7	94		
3	Recirc	19.4	0.7	95	93	1.31
4	Control	2.9	0.4	96		
4	Static	14.9	0.6	96		
4	Recirc	19.1	0.8	94	87	1.29

Table D.

Test	Starting Cells ($x10^6$)	Cell Recovery (%)	Viable Cell Recovery (%)	Efficiency (%)	Total Viable Transduced Cells ($x10^6$)	Ratio
1 (Acoustic)	8.00	97.0	87.2	35.4	2.47	1.95
2 (Acoustic)	8.00	96.7	85.8	28.8	1.98	1.56
3 (Recirc)	8.00	97.4	84.7	19.4	1.32	1.04
4 (Recirc)	8.00	94.6	82.6	19.1	1.26	1.00

Table E.

Test	Starting Cells ($x10^6$)	Cell Recovery (%)	Viable Cell Recovery (%)	Efficiency (%)	Total Viable Transduced Cells ($x10^6$)	Ratio
1 (Acoustic)	6.20	89.2	84.7	35.4	1.86	1.82
2 (Acoustic)	6.20	95.4	92.0	28.8	1.64	1.61
3 (Recirc)	6.20	91.4	87.8	19.4	1.06	1.04
4 (Recirc)	6.20	87.7	86.2	19.1	1.02	1.00

[0119] As can be seen in Table C, the use of recirculation improved transduction efficiency. The use of acoustics in addition to recirculation improved the transduction efficiency even more. Referring to Table D, the two runs with acoustics and recirculation (Runs 1 & 2) showed about 72% more total viable transduced cells compared to the two runs with recirculation but without acoustics (Runs 3 & 4). Referring to Table E, the two runs with acoustics and recirculation (Runs 1 & 2) showed about 68% more total viable transduced cells compared to the two runs with recirculation but without acoustics (Runs 3 & 4).

[0120] **Example 4:**

[0121] Three acoustic tests were performed using the acoustic chamber with recirculation as described in Example 3, but for run times of 30 minutes, 60 minutes, and 90 minutes. The acoustics were run at a frequency of 2 MHz and power of 3.5 W. Control and Static runs were also performed as described in Example 3, but for 30 minutes, 60 minutes, or 90 minutes. Table F indicates the parameters of each test. The results are shown in **FIG. 12**. As seen there, as the run time increased, the transduction efficiency compared to no acoustics improved.

Table F.

Run Time (min)	Cell concentration (10^6 / mL)	Flow Rate (mL/min)	Acoustic Volume (mL)	Total Volume (mL)	Control Volume (mL)
30	1	10	1	10	0.5
60	1	10	1	10	0.5
90	1	10	1	10	0.5

[0122] Having described several example configurations, various modifications, alternative constructions, and equivalents may be used without departing from the spirit of the disclosure. For example, the above elements may be components of a larger system, wherein other structures or processes may take precedence over or otherwise modify the application of the invention. Also, a number of operations may be undertaken before, during, or after the above elements are considered. Accordingly, the above description does not bound the scope of the claims.

CLAIMS:

1. A method for introducing foreign nucleic acids into cells, comprising:

placing the cells and nucleic acids in an acoustophoretic device comprising:

an acoustic chamber in which the cells and the nucleic acids are placed; and

an ultrasonic transducer including a piezoelectric material that can be driven to create an acoustic standing wave in the acoustic chamber; and

driving the ultrasonic transducer to create the multi-dimensional acoustic standing wave;

wherein at least the cells are retained by the acoustic standing wave, the nucleic acids being co-located with the cells to permit introduction of the foreign nucleic acids into the cells.

2. The method of claim 1, wherein the nucleic acids are in a viral vector.

3. The method of claim 1, further comprising opening pores in cell membranes of the cells prior to co-locating the cells with the nucleic acids.

4. The method of claim 3, wherein the pores are opened by electroporation, sonoporation, or by exposure to calcium phosphate.

5. The method of claim 1, wherein the acoustophoretic device further comprises a recirculation loop coupled to the acoustic chamber; and one or more of the cells or the nucleic acids are recirculated through the acoustic chamber.

6. The method of claim 1, wherein the cells are Chinese hamster ovary (CHO) cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, human cells, regulatory T-cells, helper T-cells, cytotoxic T-cells, memory T-cells, effector T-cells, gamma delta T-cells, Jurkat T-cells, CAR-T cells, B cells, or NK cells, peripheral blood mononuclear cells (PBMCs), algae, plant cells, or bacteria.

7. The method of claim 1, wherein the acoustic standing wave is a multi-dimensional acoustic standing wave, a planar standing wave, or a combination of a multi-dimensional acoustic standing wave and a planar standing wave.

8. The method of claim 1, wherein the ultrasonic transducer is driven at a frequency of about 0.5 MHz to about 20 MHz.

9. The method of claim 1, wherein the frequency of the acoustic standing wave is varied in a sweep pattern to move the cells relative to the nucleic acids.

10. A method for causing transduction of cells, comprising:

placing the cells and a viral vector comprising nucleic acids in an acoustophoretic device comprising:

an acoustic chamber in which the cells and the viral vector are placed; and

an ultrasonic transducer including a piezoelectric material that can be driven to create an acoustic standing wave in the acoustic chamber; and

driving the ultrasonic transducer to create the multi-dimensional acoustic standing wave;

wherein the cells and the viral vector are co-located by the acoustic standing wave to permit transduction of the cells.

11. The method of claim 10, wherein the cells and the viral vector are suspended in a fluid.

12. The method of claim 10, wherein the cells are Chinese hamster ovary (CHO) cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, human cells, regulatory T-cells, helper T-cells, cytotoxic T-cells, memory T-cells, effector T-cells, gamma delta T-cells, Jurkat T-cells, CAR-T cells, B cells, or NK cells, peripheral blood mononuclear cells (PBMCs), algae, plant cells, or bacteria.

13. The method of claim 10, wherein the acoustophoretic device further comprises a recirculation loop coupled to the acoustic chamber; and one or more of the cells or the nucleic acids are recirculated through the acoustic chamber.

14. The method of claim 10, wherein the ultrasonic transducer is driven at a frequency of about 0.5 MHz to about 20 MHz.

15. The method of claim 10, wherein the frequency of the acoustic standing wave is varied in a sweep pattern to move the cells relative to the viral vector.

16. A method for causing transfection of cells, comprising:

opening pores in cell membranes of the cells;

placing the cells and nucleic acids in an acoustophoretic device comprising:

an acoustic chamber in which the cells and the nucleic acids are placed; and

an ultrasonic transducer including a piezoelectric material that can be driven to create an acoustic standing wave in the acoustic chamber; and

driving the ultrasonic transducer to create the acoustic standing wave;

wherein the cells and the nucleic acids are co-located by the acoustic standing wave to cause transfection of the cells.

17. The method of claim 16, wherein the pores are opened by electroporation, sonoporation, or by exposure to calcium phosphate.

18. The method of claim 16, wherein the pores are opened before or after the cells are placed in the acoustophoretic device.

19. The method of claim 16, wherein the acoustophoretic device further comprises a recirculation loop coupled to the acoustic chamber; and one or more of the cells or the nucleic acids are recirculated through the acoustic chamber.

20. The method of claim 16, wherein the cells are Chinese hamster ovary (CHO) cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, human cells,

regulatory T-cells, helper T-cells, cytotoxic T-cells, memory T-cells, effector T-cells, gamma delta T-cells, Jurkat T-cells, CAR-T cells, B cells, or NK cells, peripheral blood mononuclear cells (PBMCs), algae, plant cells, or bacteria.

21. The method of claim 16, wherein the acoustic standing wave is a multi-dimensional acoustic standing wave, a planar standing wave, or a combination of a multi-dimensional acoustic standing wave and a planar standing wave.

22. The method of claim 16, wherein the ultrasonic transducer is driven at a frequency of about 0.5 MHz to about 20 MHz.

23. The method of claim 16, wherein the frequency of the acoustic standing wave is varied in a sweep pattern to move the cells relative to the nucleic acids.

24. The cells produced by the method of claim 1.

25. The cells produced by the method of claim 10.

26. The cells produced by the method of claim 16.

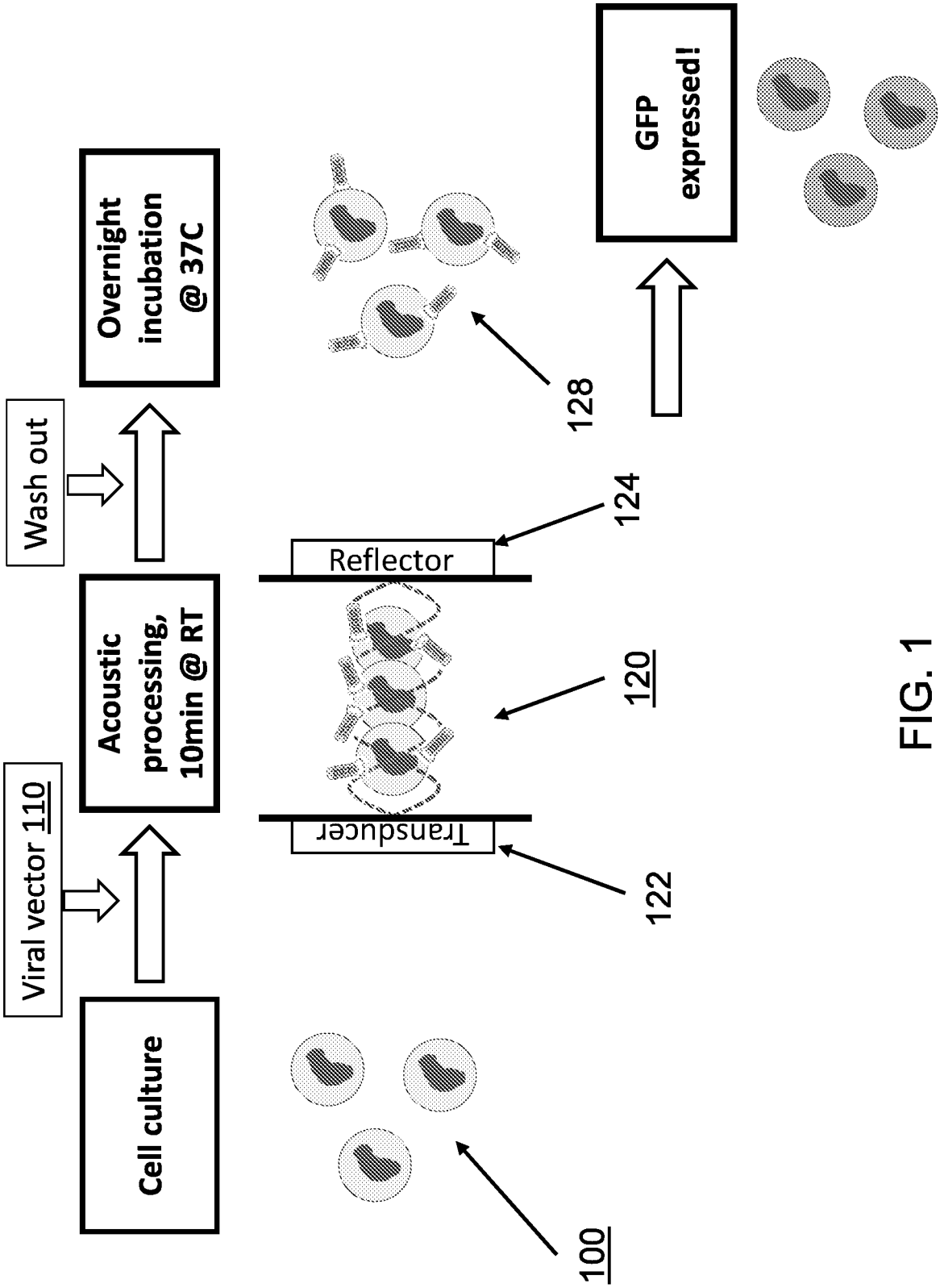
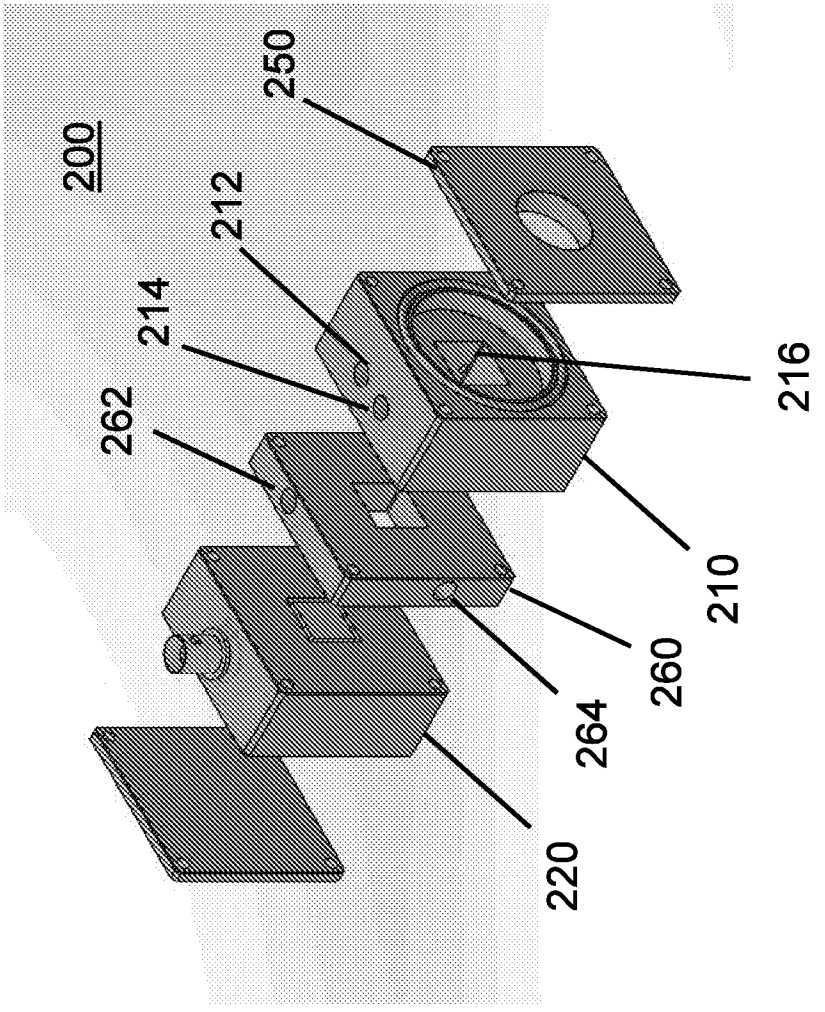

Cell culture
Viral vector 110
Acoustic processing, 10min @ RT
Wash out
Overnight incubation @ 37C
GFP expressed!
Reflector
Transducer
100
120
122
124
128

FIG. 1

FIG. 2A



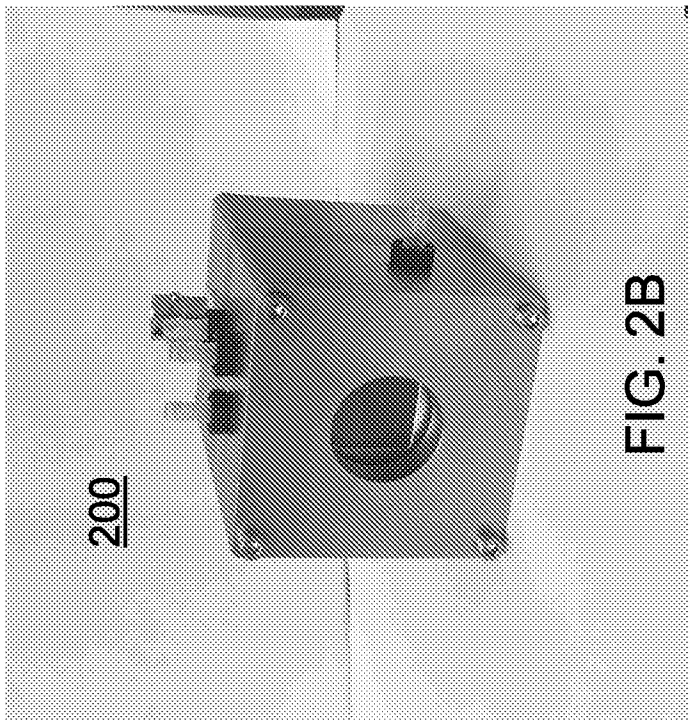


FIG. 2B

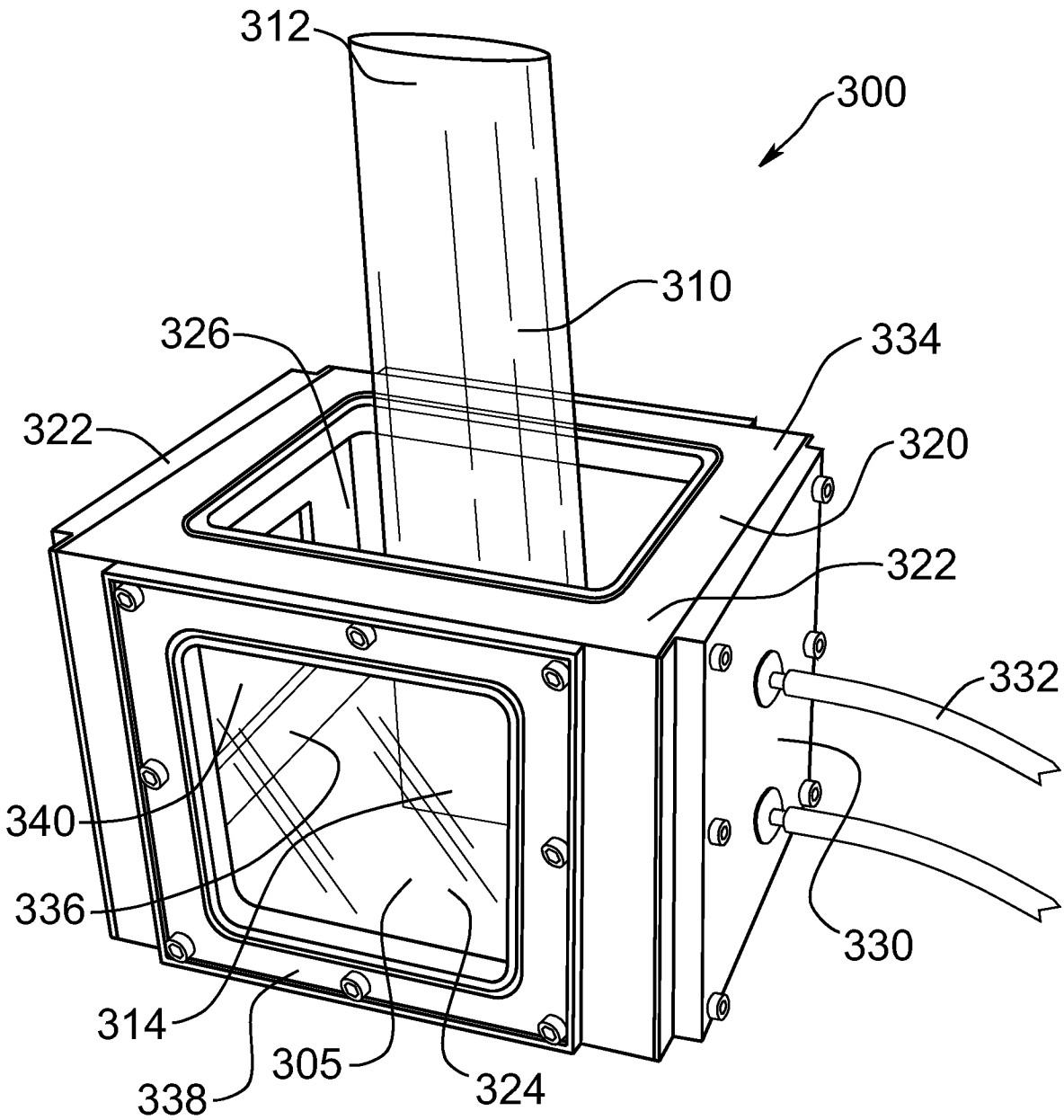


FIG. 3

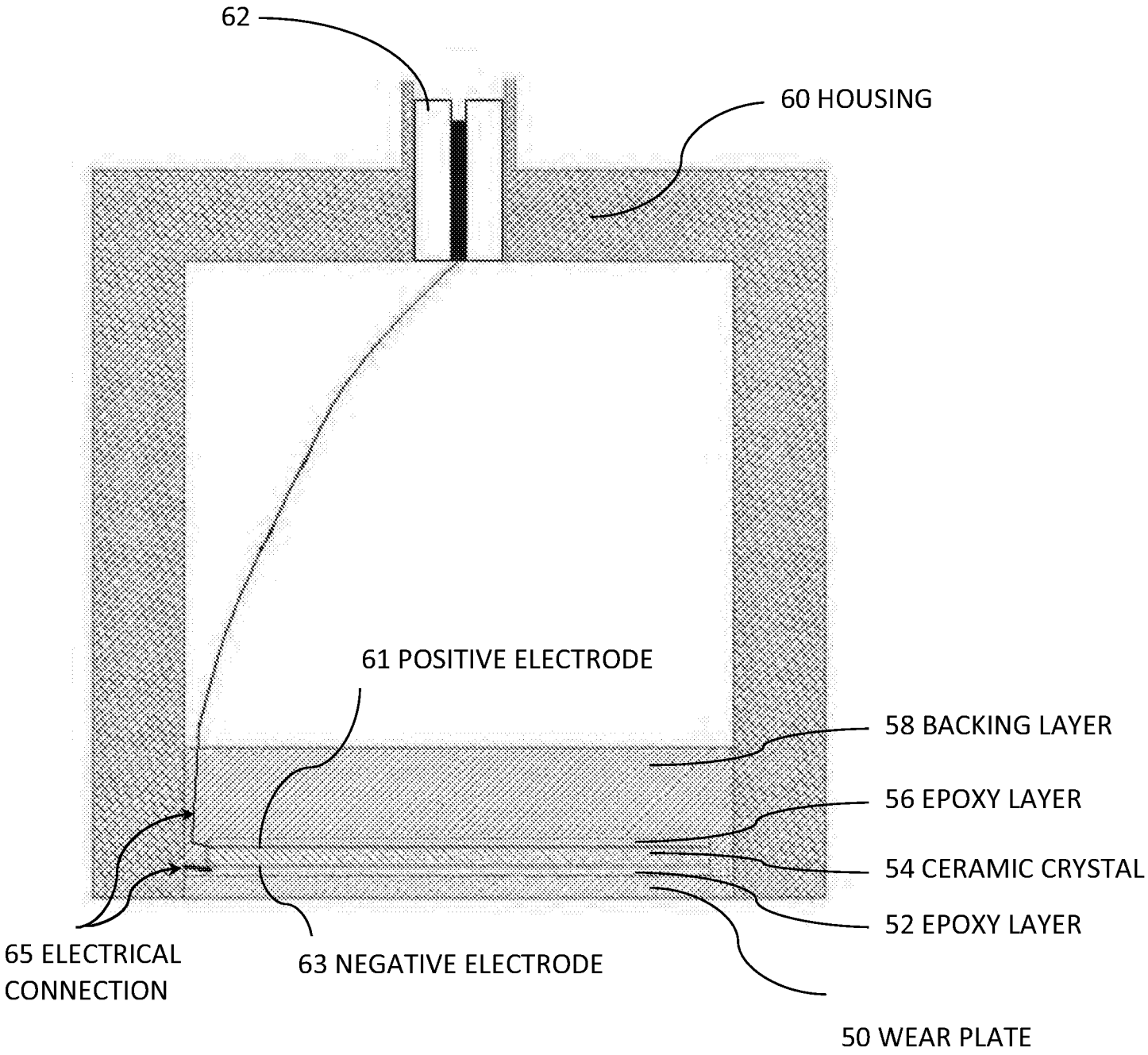

62
60 HOUSING
61 POSITIVE ELECTRODE
58 BACKING LAYER
56 EPOXY LAYER
54 CERAMIC CRYSTAL
52 EPOXY LAYER
65 ELECTRICAL CONNECTION
63 NEGATIVE ELECTRODE
50 WEAR PLATE

FIG. 4

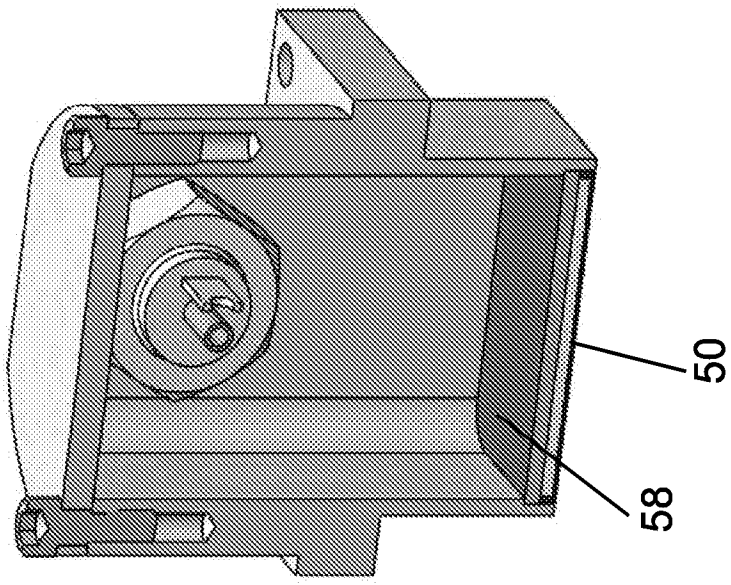


FIG. 6

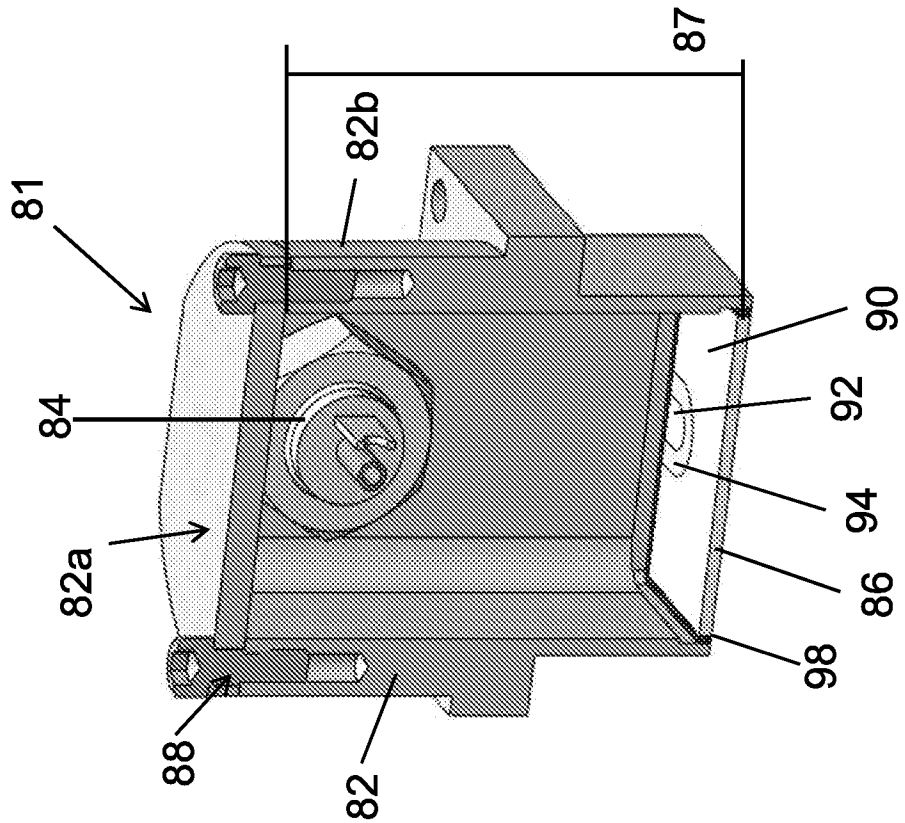


FIG. 5

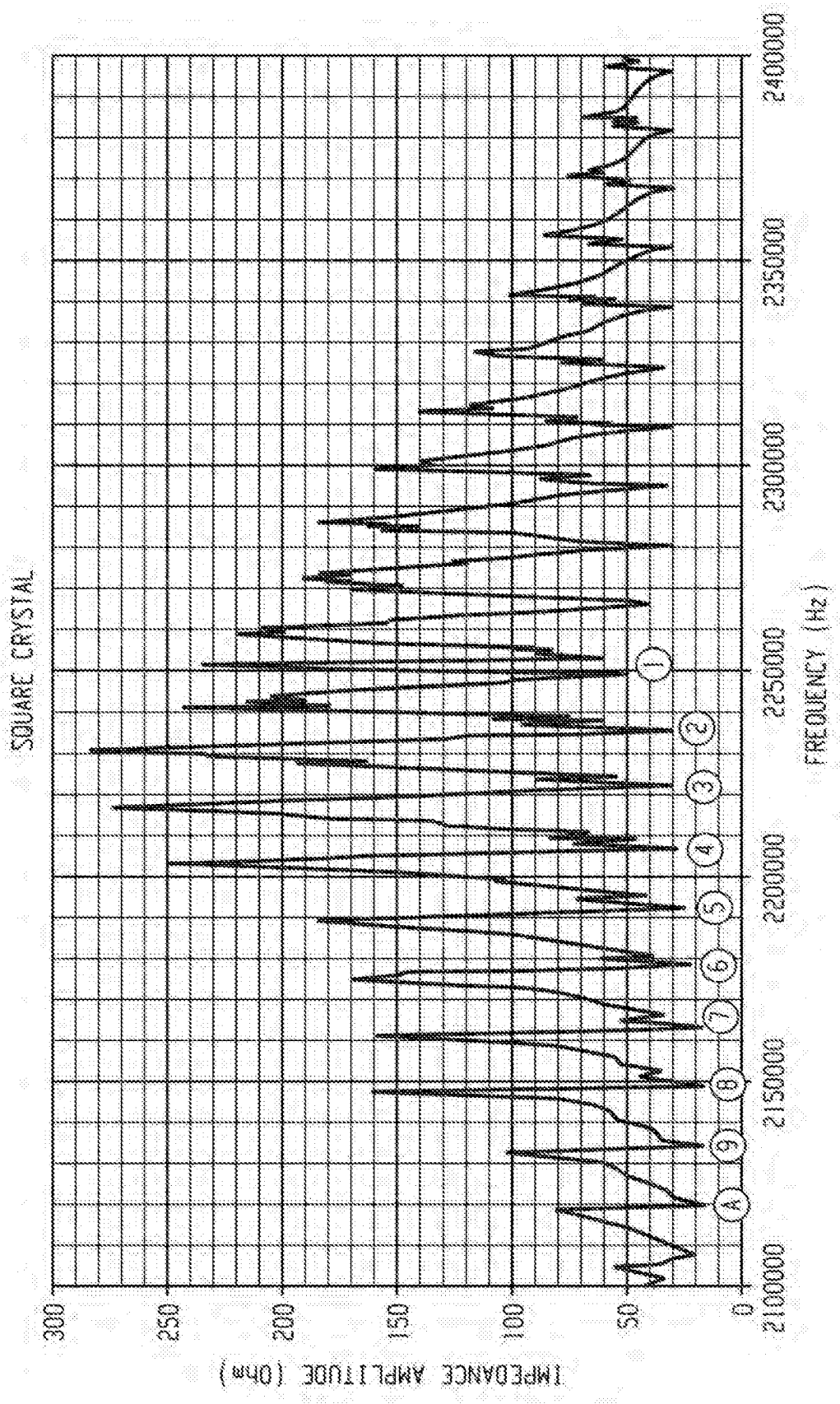

SQUARE CRYSTAL
IMPEDANCE AMPLITUDE (Ohm)
FREQUENCY (Hz)
300
250
200
150
100
50
0
2100000
2150000
2200000
2250000
2300000
2350000
2400000
1
2
3
4
5
6
7
8
9
A

FIG. 7

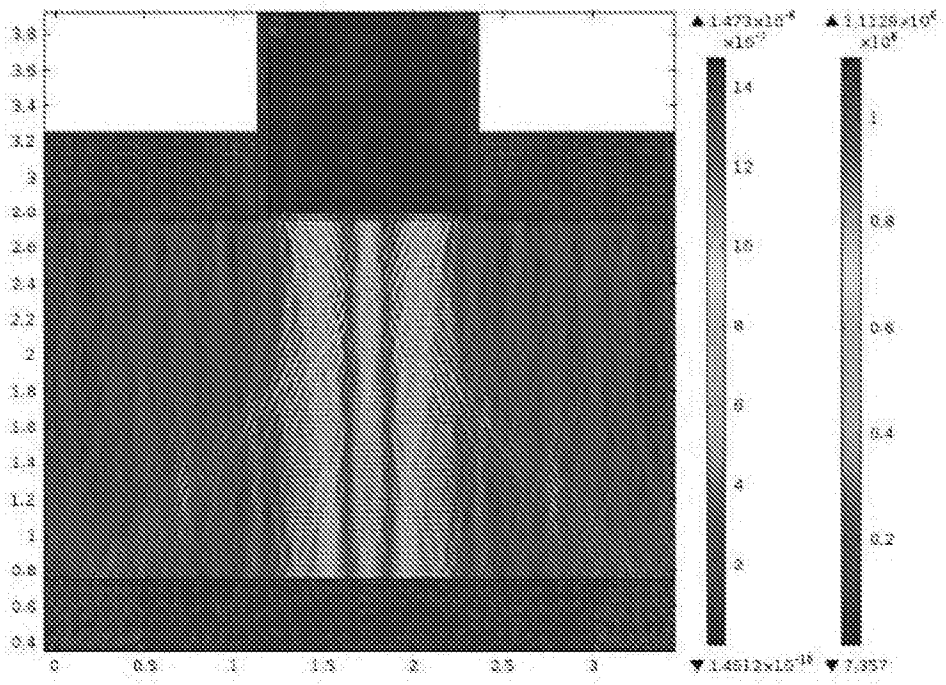


FIG. 9

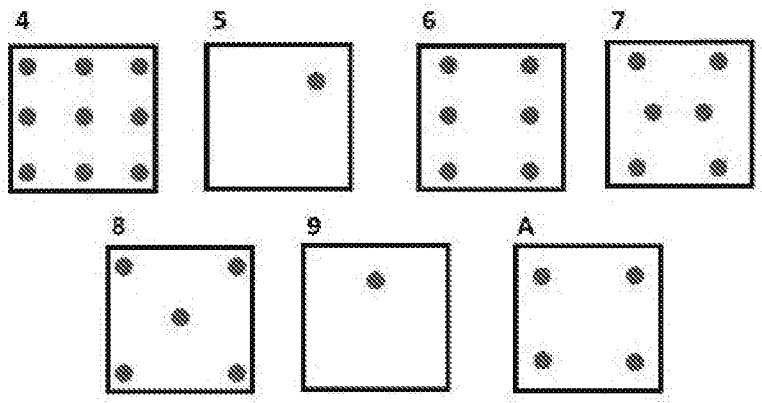


FIG. 8

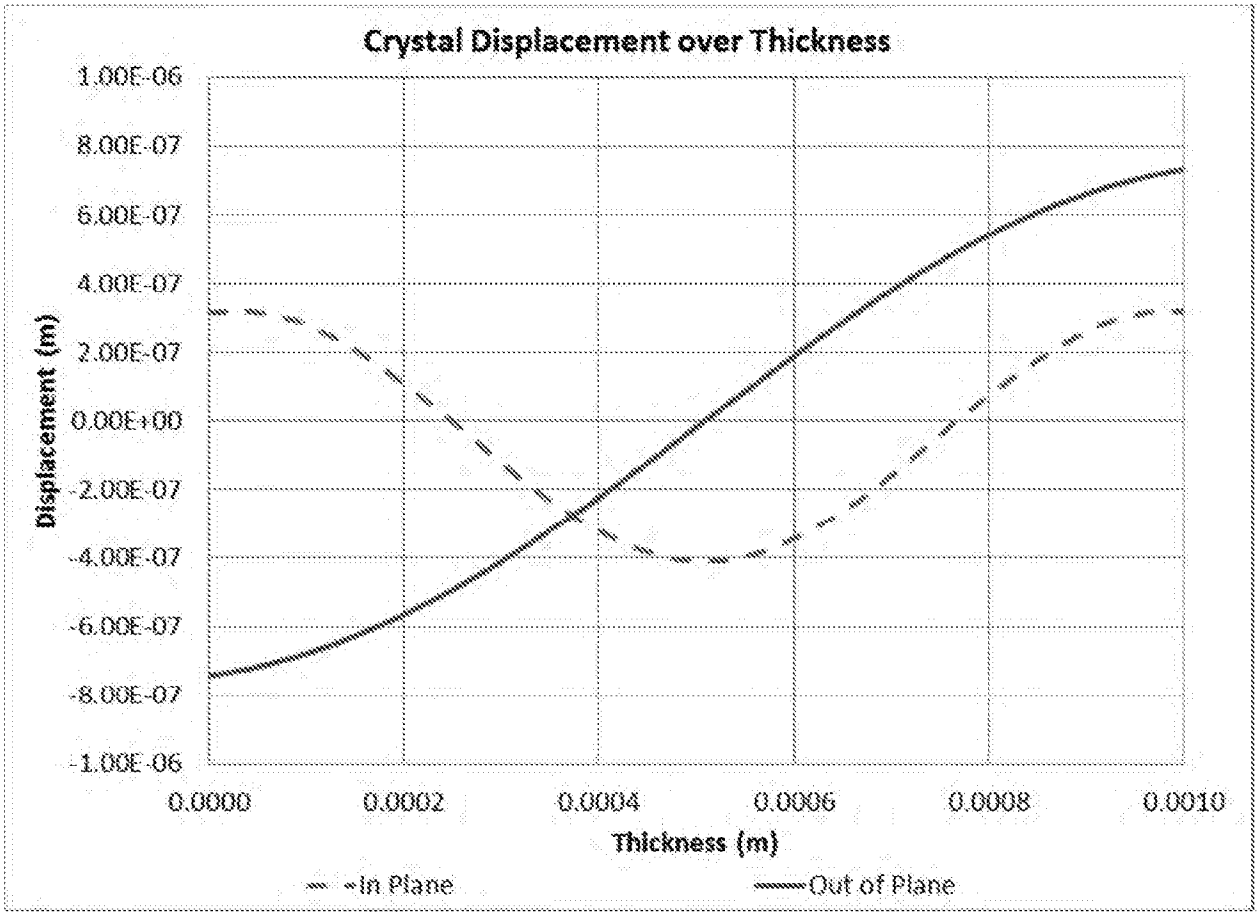

Crystal Displacement over Thickness
1.00E-06
8.00E-07
6.00E-07
4.00E-07
2.00E-07
0.00E+00
-2.00E-07
-4.00E-07
-6.00E-07
-8.00E-07
-1.00E-06
Displacement (m)
0.0000
0.0002
0.0004
0.0006
0.0008
0.0010
Thickness (m)
In Plane
Out of Plane

FIG. 10

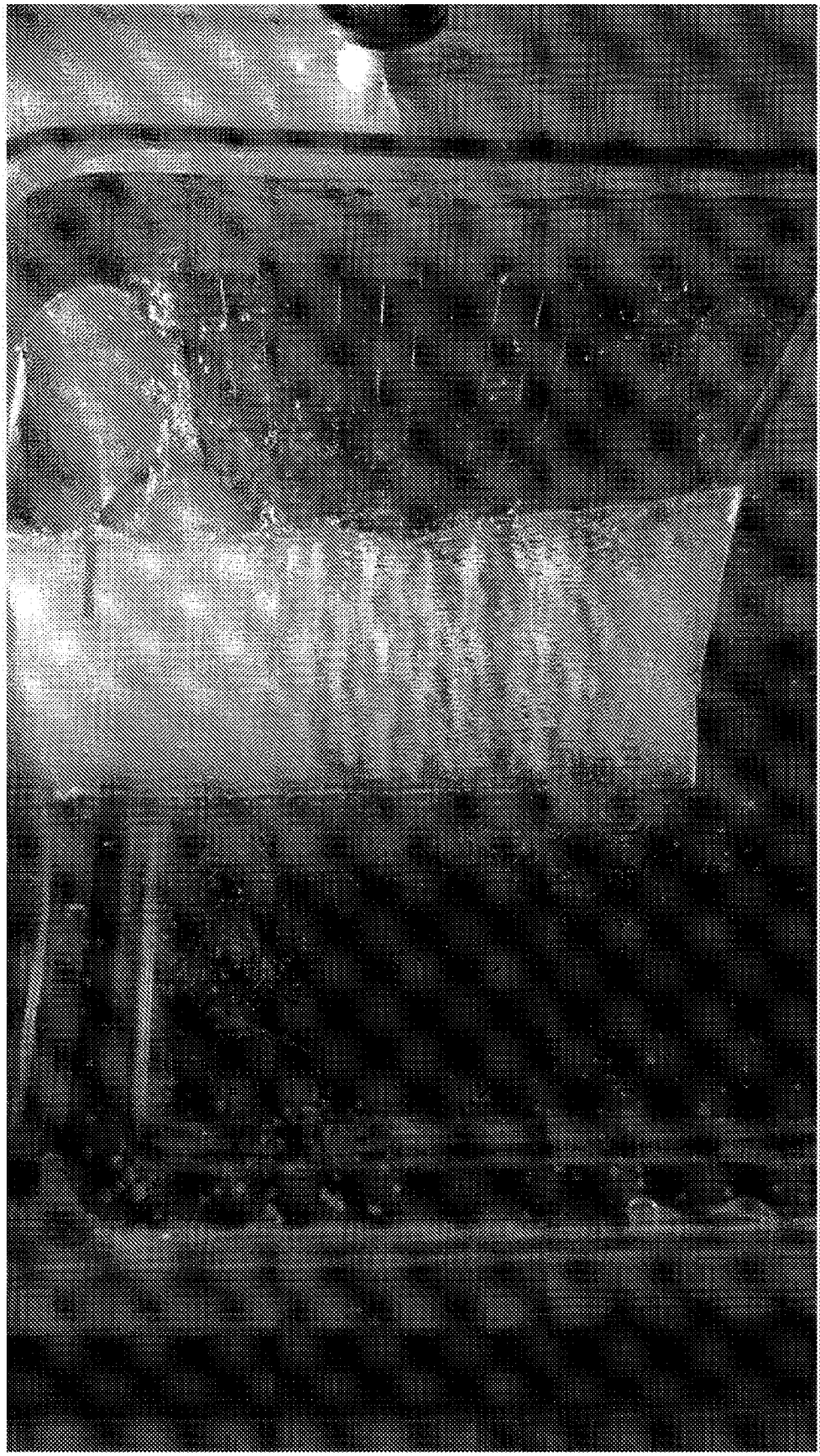


FIG. 11

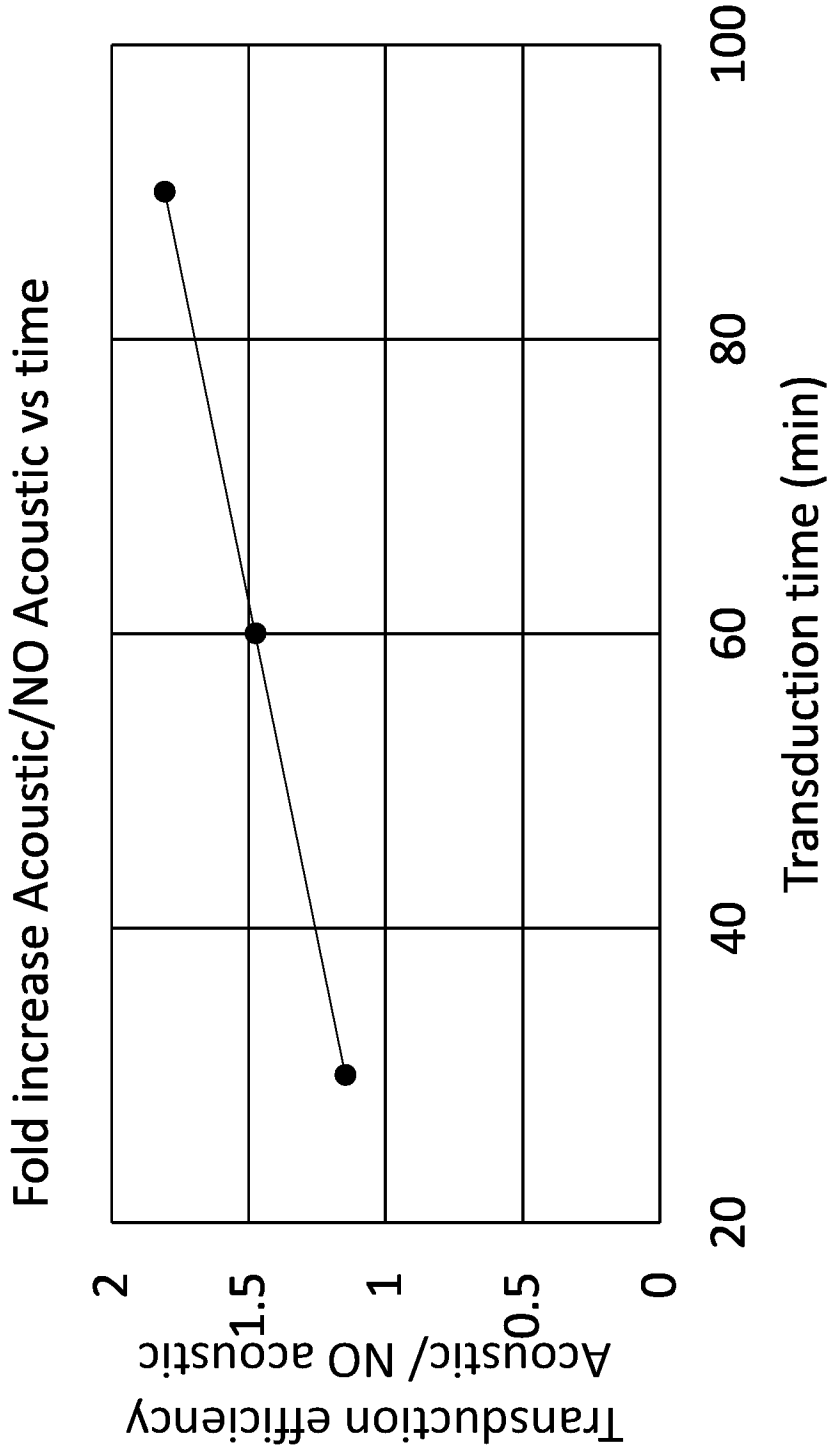

Fold increase Acoustic/NO Acoustic vs time
Transduction efficiency
Acoustic/ NO acoustic
0
0.5
1
1.5
2
20
40
60
80
100
Transduction time (min)

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/021708

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61M37/00 C12M1/42 C12N15/86
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61M C07K C12M C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/166860 A1 (PRESZ JR WALTER M [US] ET AL) 15 June 2017 (2017-06-15) paragraph [0010] - paragraph [0031]; figures 1-35	1-12
X	RESLAN L ET AL: "Transfection of cells in suspension by ultrasound cavitation", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 142, no. 2, 3 March 2010 (2010-03-03) , pages 251-258, XP026905288, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2009.10.029 [retrieved on 2009-11-06] page 251 - page 258; figures 3-5	1-12

-/--

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

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2019	22/05/2019

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

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/021708

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE ET AL: "Nonviral Transfection of Suspension Cells in Ultrasound Standing Wave Fields", ULTRASOUND IN MEDICINE AND BIOLOGY, NEW YORK, NY, US, vol. 33, no. 5, 29 April 2007 (2007-04-29) , pages 734-742, XP022052431, ISSN: 0301-5629, DOI: 10.1016/J.ULTRASMEDBIO.2006.10.015 page 734 - page 742	1-12
A	BART LIPKENS ET AL: "Macro-scale acoustophoretic separation of lipid particles from red blood cells", THE JOURNAL OF THE ACOUSTICAL SOCIETY OF AMERICA, vol. 133, 2 June 2013 (2013-06-02), page 045017, XP055162509, New York, NY, US ISSN: 0001-4966, DOI: 10.1121/1.4799371 page 45017 - pages 45017-7	1-12

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/021708

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2017166860 A1	15-06-2017	NONE	