

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 November 2009 (19.11.2009)

PCT

(10) International Publication Number
WO 2009/140701 A2

(51) International Patent Classification:
C12N 15/87 (2006.01)

(21) International Application Number:
PCT/US2009/044415

(22) International Filing Date:
18 May 2009 (18.05.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/054,070 16 May 2008 (16.05.2008) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ,
EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG,
SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR),
OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML,
MR, NE, SN, TD, TG).

Published:

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

(54) Title: DELIVERY INTO CELLS USING ULTRA-SHORT PULSE LASERS

(57) Abstract: The present invention describes the use of ultra-short pulse (USP) lasers to deliver macromolecules into cells. Pro-
vided herein are methods to introduce macromolecules such as proteins, peptides, amino acids, nucleic acids, DNA, RNA,
oligonucleotides, lipids, carbohydrates or any combinations thereof into cells having cell walls, such as bacteria, archaea,
fungi, yeast, algae, and plant cells, using an ultraviolet USP laser.



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DELIVERY INTO CELLS USING ULTRA-SHORT PULSE LASERS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to US provisional application 61/054,070, filed May 16, 2008, which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates generally to the use of lasers to deliver macromolecules into cells; and more specifically, to the use of non-thermal ultraviolet range ultra-short pulse lasers to introduce macromolecules into cells having cell walls, such as bacteria, archaea, fungi, yeast, algae, and plant cells.

[0003] DNA delivery into cells is an essential step in the genetic transformation of any organism. Various mechanical, electrical, biological or chemical methods have been developed for DNA delivery into a variety of cell types. These include heat shock transformation facilitated by certain salts (CaCl_2 , Li acetate, *etc.*), electroporation, biolistic particle bombardment, aerosol beam bombardment, cell penetration with magnetic particles, delivery with silicon carbide whiskers, microinjection, bacterial DNA transfer (*e.g.*, *Agrobacterium*), viral gene transfer, and the like. Despite the large number of available methods however, there are relatively few organisms for which highly efficient transformation methods have been developed. DNA delivery is generally inefficient and difficult in organisms surrounded by cell walls (for example, bacteria, fungi, yeasts, algae and plants). The reason for the low efficiency can be varied: for example, cell walls tend to have a small pore size and represent a formidable barrier for entry of macromolecules such as DNA, positive pressure inside the cells can limit the ability of molecules to enter the cell, and the presence of large vacuoles in the cell may predispose any macromolecules that do penetrate into the cell to be transported into the vacuole and degraded. Accordingly, there remains a need in the art for new methods to overcome any or all of these barriers.

[0004] Ultra-short pulse (USP) lasers have found wide application in many fields including medicine, manufacturing and biology. USP lasers and traditional continuous wave (CW) lasers differ fundamentally in the way they interact with matter, particularly in the ablation process (removal of material). A CW laser uses a process of linear excitation, generating substantial heat during ablation. The heat generated transfers to the

area surrounding the target, leading to melting, material reflow or tissue charring. As such, pulsed lasers, and in particular USP lasers, present many advantages over CW lasers when used for ablation. With CW lasers, thermal energy created in the process is often transferred to the area surrounding the target. By contrast, the short light pulses of USP lasers achieve ablation through non-linear effects caused by the interaction of photons with matter, and little or no heat is produced due to the short duration of the energy transfers and transformations. For example, materials can be vaporized and liquids caused to undergo phase transitions or rapid local movements by the very high peak pulse energy. Desired effects such as material expulsion from a surface, high-energy local liquid movements or phase transitions occur before thermal excitation can take place. As a result, USP lasers are the tool of choice when precise or high-resolution (on a micron scale) cuts, ablations or other transformations are required, or when sensitive materials are processed that need to be protected from thermal effects adjacent to the target sites.

[0005] US Patent Application Publication No. 2006/0141624 describes the use laser radiation for targeted transfer of molecules with a near-infrared laser beam having an emission in the wavelength range of 700 nm to 1200 nm. A similar wavelength of 800 nm has also been reported for transfection of certain mammalian cells (Tirlapur, U.K., and Konig, K. 2002. *Nature* 418:290-291). Most recently, USP near-infrared lasers have also been used to transfect various mammalian cells, for example, stem cells (Uchugonova *et al.* 2008. Targeted transfection of stem cells with sub-20 femtosecond laser pulses. *Optics Express* 16:9357-9364), mouse NIH3T3 fibroblast cells (Kaji, T. *et al.* 2007. Nondestructive micropatterning of living animal cells using focused femtosecond laser-induced impulsive force. *Appl. Physics Lett.* 91:023904-1-3), and CHO Chinese Hamster ovary cells (Stevenson, D. *et al.* 2006. Femtosecond optical transfection of cells: viability and efficiency. *Optics Express* 14:7125-7133). Various authors have described the use of ultraviolet (UV) range lasers with longer pulses, for example, 6-16 nanoseconds, to transform or transfect plant tissues, for example, by visualizing and targeting individual cells microscopically (see, *e.g.*, Weber, G. 1988. Microperforation of Plant Tissue with a UV Laser Microbeam and Injection of DNA into Cells. *Naturwissenschaften* 75:35-36; Badr, Y.A. *et al.* 2005. Production of fertile transgenic wheat plants by laser micropuncture. *Photochem. Photobiol. Sci.* 4:803-807), or by introducing foreign DNA-coated gold particles into specific cells (see, *e.g.*, Kajiyama *et*

al. 2007. Novel plant transformation system by gene-coated gold particle introduction into specific cell using ArF excimer laser. *Plant Biotechnol.* 24:315-320.

[0006] However, there remains a need in the art to expand the use of laser technologies for delivering macromolecules to cells having cell walls as described above. In particular, USP laser technologies offer distinct advantages in the field, such as minimal cell damage, that have not yet been successfully applied to cells having cell walls.

BRIEF SUMMARY OF THE INVENTION

[0007] The present invention provides methods of introducing macromolecules into cells having cell walls with a USP laser capable of delivering ultra-short ultraviolet laser beam pulses of less than 1 nanosecond.

[0008] The present invention provides method for introducing macromolecules into a cell having a cell wall, comprising: (i) contacting a plurality of cells having cell walls with a macromolecule-containing solution; (ii) exposing said plurality of cells to ultraviolet laser beam pulses of less than 1 nanosecond generated by an ultra-short pulse laser; and (iii) allowing said plurality of cells to maintain viability and/or grow under conditions that permit detection of the macromolecule.

[0009] In various embodiments, the cell is a bacterial cell, an archaeal cell, a fungal cell, a yeast cell, an algal cell, or a plant cell. In a preferred embodiment the cell is a plant cell that is present in a sample of plant tissue, which may be present in leaf disks, petioles, roots, bulbs, tubers, hypocotyls, shoots, meristematic tissues, inflorescences, reproductive cells, pollen, microspores, embryos, calli, fruit, seeds and parts or sub-parts of any of these types of plant tissues.

[0010] In various embodiments the macromolecule-containing solution contains a plurality of macromolecules selected from the group consisting of a protein, a peptide, an amino acid, a nucleic acid, DNA, RNA, an oligonucleotide, a lipid, a carbohydrate, and a combination of any thereof. The macromolecules may be histones, transcription factors, nucleolamins, RNA-binding proteins, DNA-binding proteins, DNA-replication proteins, DNA recombination proteins and DNA-repair proteins.

[0011] In a preferred embodiment the ultraviolet laser beam pulses has a wavelength of less than 400 nm. In another preferred embodiment the cell is a plant cell and said macromolecule-containing solution comprises nucleic acid molecules.

[0012] In another embodiment, the plurality of cells is placed into a translucent container, such as a plate or tube, and the container permits transmission of ultraviolet laser beam pulses through a surface of said container. In a preferred embodiment, the ultraviolet laser beam pulses are focused onto an interface between the plurality of cells and a surface of the container, such as the bottom of a plate. In another preferred embodiment, movement is at about 0.1 - 100 mm/s in a gridded pattern covering an area of about 0.1 - 10 cm². In various preferred embodiments, the ultraviolet laser beam is about 2-50 μ m in spot diameter, less than 2 μ m in spot diameter, or 0.1 – 1 μ m in spot diameter.

[0013] In various embodiments, cells are exposed to the laser beam by moving the cells through a path of said ultraviolet laser beam using a mobile support upon which a container containing the plurality of cells is positioned, or by moving the ultraviolet laser beam across the plurality of cells which remain in a fixed position. In certain embodiments movement may be continuous. In other embodiments, movement may be intermittently stopped (*i.e.*, relative movement between the ultraviolet laser beam and the plurality of cells) by stopping movement of the ultraviolet laser beam and/or said plurality of cells to turn on and/or off the ultraviolet laser beam pulses; to alter pulse number, frequency, duration, focal plane, or strength of the ultraviolet laser beam pulses; or to adjust packets of pulses for any parameters, such as those listed above and the like..

[0014] In an alternative embodiment, the cells are contacted with the ultraviolet laser beam pulses while being physically agitated, for example, by mixing, stirring, vibrating, shaking, sonicating, bubbling gases through the cells, pumping the cells into or through the container, and the like.

[0015] In certain embodiments, the macromolecule-containing solution further comprises an additive, such as a polyamine (such as spermidine or spermine), a DNA condensation agent (such as hexamine cobalt), a DNA-binding dye (such as bis-benzamide, Hoechst 33258 and Hoechst 33342, and ethidium bromide) or a combination of any thereof. In other embodiments the additive may be a poly-lysine peptide, a poly-arginine peptide, a DNA-binding protein, a peptide containing a nuclear localization signal, polyethylene glycol, dextran sulfate, polyvinylpyrrolidone, or a combination of the foregoing. In a preferred embodiment, the additive is an ionophore (such as monensin), and/or a detergent or surfactant. Similar additives may also be used as a pretreatment prior to contacting the cells with the macromolecule-containing solution.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] **FIG. 1** shows two images of cells with fluorescent nuclei indicative of successful DNA delivery into plant cells using the gel/particle approach with broadbeam laser irradiation.

[0017] **FIG. 2** shows the results of DNA introduction into plant cells in the presence of an ionophore, as measured by fluorescence (relative luciferase units) for various samples as compared to no DNA, no additive/monensin and no laser controls.

[0018] **FIG. 3** shows the results of optimizing DNA introduction into plant cells with the use of an ionophore, measured as in FIG. 2.

[0019] **FIG. 4** shows the results of optimizing DNA introduction into plant cells by varying cell incubation times with an ionophore, measured as in FIG. 2.

[0020] **FIG. 5** shows the results of optimizing DNA introduction into plant cells by varying USP laser power and packet dosage, measured as in FIG. 2.

[0021] **FIG. 6** shows the results of optimizing DNA introduction into plant cells by varying detergents and surfactants, measured as in FIG. 2.

[0022] **FIG. 7** shows the results of optimizing DNA introduction into plant cells by varying USP laser frequency and speeds of irradiation while keeping the pulse dosage constant, measured as in FIG. 2.

[0023] **FIG. 8** shows further results of optimizing DNA introduction into plant cells by varying detergents and surfactants, measured as in FIG. 2.

[0024] **FIG. 9** shows the results of optimizing DNA introduction into plant cells by varying ionophores, measured as in FIG. 2.

DETAILED DESCRIPTION OF THE INVENTION

[0025] It is an object of the present invention to provide methods for the introduction of macromolecules in cells having cells walls through the use of ultraviolet range USP lasers. Irradiation of cells or tissues with USP lasers can cause many types of cellular changes, particularly on the cell surface where laser irradiation is likely to have the greatest impact. Cell surface changes can include punctures or holes in cell walls and cell membranes that directly lead to DNA take-up or other physical cell surface changes as a consequence of laser irradiation that result in DNA uptake into the cell. Accordingly, the detailed description of the invention provided herein provides the laser properties and setup, as well as particulars relating to cells and their properties, and

methods for irradiating cells with lasers, that can result in delivery into cells with cell walls, in particular plant and algal cells.

Types of molecules delivered

[0026] The present invention relates to the delivery of any type of molecule to cells or tissues. The methods described herein may be especially useful for delivery of nucleic acids, such as DNA or RNA. In many parts of the description we refer to DNA delivery or uptake. DNA is merely used as an example, and the descriptions should be understood to cover delivery of any type of molecule into a cell or specific cellular compartment.

[0027] Of particular interest is the delivery of DNA or RNA molecules together with or complexed to other molecules such as peptides or proteins or other molecules as provided herein. Association of DNA or RNA with these molecules may enhance stability of the DNA or RNA within the cell or target its movement into the correct cell compartment such as the nucleus. DNA and RNA molecules in the cell are naturally bound to proteins (histones, transcription factors, nucleolamins, RNA-binding proteins, DNA-binding proteins, DNA-replication proteins, DNA recombination proteins, DNA-repair proteins, *etc.*) and delivery of DNA or RNA molecules complexed with such peptides or proteins may enhance the function of the DNA or RNA molecules once inside the cell, such as their ability to be replicated, transcribed, translated, integrated into the genome, or converted into an active chromatin state.

[0028] In contrast to other delivery methods which may involve harsh conditions that are used to treat the DNA or RNA, USP laser-driven delivery can be effective in media similar or identical to the growth media used to culture cells, or in buffered solutions that promote DNA, RNA and protein stability, implying that DNA or RNA complexes with other molecules may be stable in the course of laser irradiation and delivery.

General laser setup and orientation

[0029] Cells to be irradiated can be contained in any vessel or container, including, but not limited to, dishes, tubes, and transparent tubing. Use of containment vessels with transparent or translucent surfaces, such as walls, lids or bottoms, allows laser irradiation through the vessel walls, lids or bottoms. The laser beam can be positioned so that it strikes the sample container in a horizontal or vertical manner, or any intermediate angle.

[0030] Laser irradiation can be performed with stationary or moving target cells, or with a stationary or moving laser beam. The advantages of moving target cells or moving laser beam are that the laser can expose a large number of cells when using movement than with stationary cells or laser beams. Cells may be moved by moving the container and/or platform supporting the container, or by physically agitating the cells, for example, by mixing, stirring, vibrating, shaking, sonicating, bubbling gases through the cells, pumping the cells into or through the container, and the like.

[0031] USP lasers may be programmable to be operable in a mode that produces continuous pulses, or with defined packets of pulses followed by a defined interval without pulses. Packets of pulses can range from a single pulse per packet to billions of pulses per packet. Specific pulse settings of the laser, involving delivery of specific numbers of laser pulses of defined energy may be required to enable DNA delivery into specific cell types. Similarly, manipulation of the cells can be programmed in regard to the laser allowing for the use of multiwell plates and the simultaneous processing of large numbers of samples (high-throughput). These parameters and laser settings may be general, with similar settings allowing delivery into cells from a variety of organisms, or may be specific to each organism or cell type.

Beam width

[0032] Beam width is a critical parameter influencing the effect of laser irradiation on cells. Plant cells are typically 10-50 μm in diameter but can be smaller or larger depending on the species, tissue and developmental stage. For the purposes of delivery into cells, beam width can be divided into two general categories, broad beams and narrow beams.

[0033] Broad beams: $> 2 \mu\text{m}$ in spot diameter; beams of this width have a higher likelihood of killing cells when used to puncture holes in cell surfaces; as a result, these beam widths are not suited for puncturing holes in cells, but can be used to achieve DNA delivery into cells via 3 mechanisms;

- a) laser-induced changes in cell surfaces that lead to DNA uptake by the cells without making a hole in the cell surface;
- b) DNA delivery into cells via secondary effects, by inducing a number of optical phenomena in the liquid surrounding the cells, including optical breakdown, plasma jets, shockwaves, rapid local movements of liquid or gases, any of

which can either puncture cell surfaces or cause other changes in cell surfaces to achieve take-up of DNA by the cell; and

- c) DNA delivery into cells via particles that are impacted into the cell surface or cell interior by broad-beam irradiation of the particles and as a consequence of the secondary effects (see also the section on particles below).

[0034] Broad beams can also be used to irradiate cells through a material that blocks laser light from reaching a portion of the sample and allows it to reach another portion, or a material that channels the laser light onto a specific location on the sample; this type of setup would effectively narrow the broad beam to a smaller width suitable for micron-scale changes in cells or cell surfaces.

[0035] Narrow beams (micropuncture): $\leq 2 \mu\text{m}$ in spot diameter; beams of this width are sufficiently small that cell survival is likely after cell puncture; as a result these beam widths are suitable for micropuncture approaches which rely in the DNA diffusing into the cell interior through a laser-induced hole. Ideally, beams are $0.1 - 1 \mu\text{m}$ in spot diameter.

Wavelengths and frequency

[0036] Any visible or non-visible wavelength of light may be suitable for irradiating cells to achieve DNA delivery. Specific wavelengths of interest include but are not limited to 388, 517, 776 and 1552 nm. Wavelengths between 200 nm and 1600 nm may be suitable to induce DNA delivery into cells. Certain wavelengths may work better for some approaches or cell types than others. In particular, invention methods described herein have found that laser pulses in the ultraviolet range (UV), *i.e.*, less than 400 nm, and in particular, 300-400 nm, were best for micropuncture of cells using narrow beams.

[0037] Frequency as used here applies not only to the frequency of light, a function of its wavelength, but also to the frequency of laser pulses. The frequency of laser pulses can range between 1 Hz to 1000 kHz or above 1000 kHz, or any intermediate frequency.

Cell types

[0038] The methods described in this invention can be applied to any organism or cells, in particular, those with a cell wall. The latter include bacteria, archaea, fungi, yeasts, algae, plants and any cells or tissues from any of these organisms, including protoplasts (cells stripped of their cell walls). Plant cells and tissues can include but are not limited to leaves including leaf disks, petioles, roots, bulbs, tubers, hypocotyls, shoots, meristematic tissues, inflorescences, reproductive cells, pollen, microspores,

embryos, fruit, seeds calli, and any fragments or pieces or developmental stages thereof, or protoplasts derived from such cells. The same methods may also be applied to cells that do not naturally contain cell walls, including mammalian, avian or insect cells, or certain algae or other microbes that naturally lack cell walls

[0039] It is a specific object of the present invention to provide methods that can successfully be applied to cells having cell walls. The materials in a cell wall vary between species, and in plants and fungi also differ between cell types and developmental stages. In plants, the strongest component of the complex cell wall is a carbohydrate called cellulose, which is a polymer of glucose. In bacteria, peptidoglycan forms the cell wall. Archaeal cell walls have various compositions, and may be formed of glycoprotein S-layers, pseudopeptidoglycan, or polysaccharides. Fungi possess cell walls made of the glucosamine polymer chitin, and algae typically possess walls made of glycoproteins and polysaccharides. Unusually, diatoms have a cell wall composed of silicic acid. Often, other accessory molecules are found anchored to the cell wall. It is these very features and components of cell walls that have been overcome by the methods of the present invention to allow the introduction of macromolecules into these cell types.

Sample mixing or agitations

[0040] To expose as many cells in a sample simultaneously to a laser, the sample may be irradiated while mixing or agitating the sample so that a large number of cells pass in front of the focused laser beam in the course of the irradiation. Methods of mixing or agitation include but are not limited to vibration, shaking, stirring (with any of a number of mechanisms including mechanical stirring, stirring with a magnetic stir bar, *etc.*), sonication or bubbling gases through a liquid suspension of the cells.

Alternatively, cells can be passed through transparent tubing in the path of a focused laser, exposing the cells to the laser as they pass through the tubing. The tubing can be of an material that is partially transparent to the laser light.

Laser or sample scanning/tracking movements

[0041] To expose a large number of cells to laser light, scanning and tracking movements can be executed in a manner that the laser beam moves across a sample in a random or non-random path. The movement of the laser beam across a sample can be achieved by moving the laser across a stationary sample, or moving a sample across a stationary laser beam, or a combination of the two. Movements may be programmed

into a motion-control system, allowing systematic scans of a sample in a grid or other pattern. During the movement, the laser may be irradiating the sample continuously (with continuous streams of laser pulses at the set frequency), or may deliver packets of pulses separated by intervals of no irradiation. The movement may be continuous, or may include stops. Laser irradiation may be limited to the periods of movement, or to the stops in a discontinuous system of movements, or a combination of the two.

Plane of laser focus and motion of the laser focal plane in the Z axis

[0042] When tracking a laser across a sample, and/or when doing individual cell irradiations, it may be difficult to ensure that the laser is focused in the appropriate plane for optimal effect on the cell and for optimal DNA delivery. This optimal plane of focus could be outside the cell or tissue, on the surface of the cell or tissue, or underneath the surface of the cell or tissue. To ensure that a sufficient number of cells are irradiated at the optimal plane of focus, movements of the laser or of the sample or of both can be employed that adjust the distance of the plane of focus of the laser from the cell, or its distance from the wall of the containment vessel. For example, at regular intervals during tracking of the laser across a sample, or movement of a sample through a laser path, the focal plane of the laser can be shifted towards or away from the sample or containment vessel. These shifts can be in regular or irregular increments, unidirectionally (*i.e.*, either towards or away from the sample) or both towards and away from the sample. Changes in the focal plane can be performed in a manner that the focal plane describes a wavelike, stepwise back-and-forth or zigzag movement towards and away from the sample, so that some irradiations will occur with the focal plane closer to or deeper within the sample and others with the focal plane further away from the sample or less deep into the sample, while executing continuous or spotwise irradiations. In this manner at least some of the irradiated cells may be in the optimal position with respect to the laser focal plane for most efficient molecule delivery into the cell. The movement of the laser's focal plane can occur in a continuous or stepwise fashion, accompanied by continuous irradiation (continuous streams of laser pulses at the set frequency) or by packets of pulses separated by intervals of no irradiation, or any combination thereof. The movement may occur between or during packets of laser pulses or a combination thereof. One of skill in the art could readily optimize such parameters.

Multiple irradiations of a single position in a sample

[0043] As noted above, the laser can be operated in a manner that the laser and the sample are stationary during irradiation, so that no deliberate movement occurs of the sample with respect to the laser beam during irradiation. As a result, one or more identical or different laser pulses or pulse packets can be delivered to the same location on the sample. The different pulses or pulse packets can be separated by time intervals ranging from picoseconds to several minutes. They can be of the same power, frequency and wavelength, or different in any of these parameters. In addition, the focal plane of the laser can be moved towards or away from the sample between different irradiations onto the same spot in the sample. The result may be a combination of identical or different laser impacts on the cell that together achieve an optimal modification of the cell surface and/or cell interior for delivery of DNA or the molecule of interest.

Use of particles

[0044] Particles of any size or composition can be used to facilitate laser-mediated DNA delivery via direct or indirect interaction with cells. Examples of particles that can be used include but are not limited to: gold, platinum, tungsten, silicon carbide, tungsten carbide, aluminum oxide, silicon dioxide, latex, polystyrene, melamine, diamond or carbon. Particles can be large compared to cell diameter (*i.e.*, $> 2\mu\text{m}$ for eukaryotic cells) or small compared to cell diameter (*i.e.*, $\leq 2\mu\text{m}$ for eukaryotic cells); particles can be uniform or non-uniform in size, regular or irregular-shaped. The particles can also be needle-shaped such as silicon carbide whiskers or carbon nanotubes.

[0045] Exemplary variations of the use of particles include the following:

[0046] Particles may be present in a cell sample that is not being mixed during irradiation. In this case, the particles would be relatively stationary with respect to the cells during irradiation. This use of particles includes layering particles on top of, adjacent to or below a sample for irradiation.

[0047] Particles may be present in a cell sample that is being mixed during irradiation. In this case, the particles would be in suspension with the cells and moving with respect to the cells during irradiation.

[0048] Particles may be bound to the cell exterior prior to irradiation. In this case, the particles may absorb laser light more efficiently than the cells, and increased absorbance of laser light by the bound particles may cause changes in the cell surface at

or adjacent to the site where they are bound. Alternatively, particles may reflect or block laser light from hitting the cells at the place where they are bound.

[0049] Hydrogel irradiation: a thin transparent layer of a viscous or gelled material present above, adjacent to or below a cell sample, with particles present between the gel and the cells is irradiated in a manner that the particles are moved into the cell sample during irradiation.

Cell staining

[0050] Dyes can be used for staining cell prior to irradiation. The dye can have various effects as follows.

[0051] The dye can absorb laser light, concentrating laser light absorption to the stained cell or cell part (*e.g.*, cell surface). For example, cell surface staining may cause preferential absorption of laser light by the cell surface, leading to increased cell surface changes compared to changes in other cell structures, or to an increase in the non-linear effects caused by laser in the areas where laser light is absorbed more strongly.

[0052] The dye can absorb laser light, thus protecting a cell or parts of the cell from deleterious effects of irradiation. For example dyes used to stain the cytoplasm, vacuole or vacuole membrane (tonoplast) may protect those compartments from laser light and prevent laser penetration into the cell.

[0053] The dye can absorb laser light and emit light of a different frequency, leading to changes in the cell or part of the cell where the dye is present caused by emission of the new frequency of light.

[0054] The dye can be applied to cells before irradiation and excess dye left in solution, or excess dye can be washed away prior to irradiation, or dye can be applied during irradiation.

Use of additives

[0055] Additives can be included in the cell suspension (for example, as a pretreatment prior to contact with the macromolecule-containing solution), the macromolecule-containing solution added to the cells, or both, that stimulate macromolecule uptake into cells, *e.g.*, increase DNA stability in the course of the irradiation or in the course of DNA transport through the cell into the nucleus. Examples of additives are as follows.

[0056] Compounds that increase DNA stability include but are not limited to polyamines (*e.g.*, spermidine, spermine), DNA condensation agents (*e.g.*, hexamine cobalt), DNA-binding dyes (*e.g.*, bis-benzamide, Hoechst 33258 and Hoechst 33342, ethidium bromide), polyethylene glycol, dextran sulfate, and polyvinylpyrrolidone.

[0057] Compounds that increase DNA transport to the nucleus include but are not limited to polyamines (*e.g.*, spermidine, spermine), proteins or peptides rich in lysine or arginine, or DNA-binding proteins or peptides containing nuclear localization signals.

Altered physical conditions

[0058] Altered physical conditions can be used before, during and/or after irradiation of cells to increase the rate of DNA delivery. The physical conditions that can be manipulated include temperature, pressure or osmotic strength of the medium. The conditions can be changed in either direction from normal, *e.g.*, pressure can be increased or decreased, *etc.* The changes can be made before, during or after irradiation or a combination thereof. Other physical parameters can be manipulated also including other physical cell treatments such as sonication, agitation or electroporation, before, during or after laser irradiation.

Cell treatments

[0059] Cells can be treated with agents before, during and/or after irradiation to achieve macromolecule uptake by the cell in response to laser irradiation. Treatments can include any chemical, biological or agent that alters the cell surface, cell interior or cell organelles in a manner that uptake and transport (*e.g.*, DNA to the nucleus) are increased. For example, cell surfaces can be made more or less thick, porous, permeable, fluid, conductive or leaky by treatment with various agents that modify cell walls or cell membranes. Examples of such agents are detergents, ionophores, surfactants, lipids, membrane-inserting hydrophobic compounds or pore-forming peptides. In particular, treatment of cells with mild detergents or ionophores are promising approaches that can lead to DNA delivery into a cell when combined with laser irradiation. Various examples are cited that present data of such cell treatments resulting in higher rates of DNA delivery into the cell. Similar approaches may work for other cell types such as bacteria, archaea, fungi, yeasts, algae, plants and any cells or tissues from any of these organisms, including protoplasts, as well as mammalian or avian or insect cells.

[0060] Organelle function, such as the function of the vacuole and/or nucleus can be affected by treatment with agents that disrupt vacuole and/or nuclear structure or function. Membrane composition can be altered by treatment with agents that bind to or inhibit the biosynthesis of membrane components.

[0061] Table 1 summarizes various approaches for the use of USP lasers in experiments aimed at delivery of macromolecules to cells as described herein. Parameters relating to types of molecules delivered, organism types, cell types, laser setup and properties, cell positioning, handling and irradiation, additives, physical parameters and cell treatments are listed.

Table 1: Summary of approaches for USP laser-directed delivery of macromolecules into cells

Category	Invention or Method	Brief Description
Types of molecules delivered	Macromolecules	DNA, RNA, protein, polysaccharide, lipid, or complexes or combinations of any of these
	DNA or RNA molecules complexed with other molecules	DNA and RNA molecules can be complexed with other molecules prior to delivery into cells. The other molecules can include polyamines, peptides, proteins, lipids, surfactants, detergents or any other substance that binds to DNA or RNA directly or indirectly
	Small molecules	Any small molecule that does not naturally penetrate into a cell, or whose penetration rate into cells or final concentration within cells is low or otherwise sub-threshold
General laser setup and orientation	Horizontal Irradiation	Irradiating samples horizontally from the side of the containment vessel
	Vertical irradiation	Irradiating samples vertically from above the sample or from below the sample through the translucent lid or bottom of the containment vessel
	Irradiation of cells with laser in packet mode	Packet mode is a programmable mode consisting of a defined number of laser pulses followed by a defined number of non-pulses; when using this mode while scanning across a tissue can produce short bursts of laser light at specific locations and intervals between which the laser is off; is equivalent to punching holes in cells and then scanning to the next location
	Cells and laser stationary during irradiation	Laser beam in fixed position relative to cell sample
	Cells and/or laser moving during irradiation	Cells moving relative to one another, or moving relative to laser beam
Beam width	Broad-beam irradiation	Irradiation of cells with beam diameter large compared to cell diameter (i.e. $> 2\mu\text{m}$ for eukaryotic cells) for delivery via secondary effects (i.e. laser-induced optical breakdown, shockwaves, liquid, gas or plasma movements that puncture or otherwise affect cell surface, or cause other changes to the cell or cell surface that facilitate or induce molecule take-up by the cell)
	Broad beam gridded irradiation	Broad beam irradiation through an ultrathin, micro-machined plate grid that allows laser photons to pass through some areas of the grid while blocking it in other areas
Wavelength	Narrow-beam irradiation (micropuncture)	Irradiation of cells with beam diameter small compared to cell diameter (i.e. $\leq 2\mu\text{m}$ for eukaryotic cells) for delivery via holes punctured in cells; this is achieved by increasing magnification/increase of objective lense to decrease laser beam spot size. Decreasing spot size may allow more precise method for penetrating cell wall with little to no effect to cell viability
	Wavelengths used	398, 517, 775, 1552 nm including all wavelengths in between, and wavelengths as low as 200 nm
Cell types	Near UV wavelength irradiation	Use of wavelengths below 400 nm may be best for micropuncture of cells
	Classes of organisms	Bacteria, archaea, fungi, yeast, algae, plants and any cells or tissues from any of these organisms including protoplasts (cells stripped of their cell walls), also cell wall-free organisms such as mammalian cells, avian cells, reptilian cells, amphibian cells or insect cells
	Plant cell types	Callus, suspension cells, embryogenic callus, any explants (plant tissues or organs or pieces thereof) or developmental stages thereof, including but not limited to leaves including leaf disks, petioles, roots, bulbs, tubers, hypocotyls, shoots, meristematic tissues, inflorescences, reproductive cells, pollen, microspores, embryos, fruit, seeds; protoplasts from any of these tissues
Sample mixing or agitations	Size of cells	Cells can range in size from 0.1 to $>500\mu\text{m}$ in any dimension
	Mixing or agitating cells during irradiation	Mixing, agitating or circulating cells in a containment vessel front of a laser beam by stirring cell suspension mechanically or with magnetic stirbar driven by exterior magnetic stirrer or other means, by vibrating or shaking the containment vessel (i.e. via a vibrating or shaking device directly or indirectly attached to the containment vessel), by sonicating the cell suspension or containment vessel, or by bubbling a gas through the cell suspension
Laser or sample scanning/ tracking movements	Circulating cells through irradiation zone inside transparent tubing	Cells/ molecule are moved or circulated through a capillary tube passing through laser irradiation zone, allowing cell exposure to the laser as they pass through the tube
	Automated tracking of laser or of sample during irradiation	Laser is programmed in X,Y,Z space to track across stationary cells/tissue or across the containment vessel wall with stationary cells; laser scans cells at a height appropriate for molecule uptake in the largest number of cells possible, scanning can be achieved using moving laser beam or moving sample
	Stop-Shoot-Scan	Laser is programmed in X,Y,Z space to track across stationary cells/tissue or across the containment vessel wall with stationary cells with the ability to stop, irradiate, and then track in X,Y,Z space further while repeating this pattern at designated intervals.
Use of particles	High-throughput multiwell laser transformation	Cells of various type and conditions are prepared in multiwelled plates i.e. 384 well plates and irradiated with a program specific to that particular plate in the X,Y,Z axes to achieve automated irradiation of every well. Laser conditions may also be customized to individual wells
	Irradiation in presence of micro- and nanoparticles	Use of particles made of any material i.e. latex, diamond, silicon carbide, tungsten carbide, melamine, aluminum oxide, SiO ₂ , latex (unmodified or modified), tungsten, platinum or gold for direct and indirect interactions with cells; particles can be large compared to cell diameter (i.e. $> 2\mu\text{m}$ for eukaryotic cells) or small compared to cell diameter (i.e. $\leq 2\mu\text{m}$ for eukaryotic cells); particles can be uniform or non-uniform in size, regular or irregular-shaped
	Irradiation with particles co-suspended with cells	Circulating or mixing particles in suspension with cells; particles when irradiated can generate secondary effects by breaking off and striking cells, imbedding into cells or other effects leading to particle contact with the cells or particle entry into cells following laser irradiation
Cell staining	Irradiation with particles stationary with respect to cells	Stationary cells in a containment vessel, with particles layered on top, mixed within or layered underneath cells or tissue sample; irradiation proceeds into particle layer or cell layer
	Coating cells with particles prior to irradiation	Cells are coated with particles that adhere to cell surface and then cells are irradiated; particles, particularly opaque ones, will absorb laser light more strongly than the cells, causing local effects on cell surface leading to molecule uptake; alternatively, bound particles may reflect or block laser light from reaching the cells at the place where they are bound
	Hydrogel irradiation	Irradiation of molecule and/or particles deposited or bound on layers of gelled material deposited on vessel base allowing for propulsion of molecule and/or particles into cells after irradiation of gelled material or interface of gelled material and particles or cells
Use of additives	Staining cells exterior with dyes	Staining cell exterior with dyes to increase laser absorption by cell surface, compared to laser absorption in unstained and more translucent cells; opaque cell surface absorbs laser light more efficiently resulting in holes or other changes in cell surface without destruction of cell interior, more efficient laser effects including absorption of laser pulses, non-linear effects and optical breakdown effects close to absorbent cell surface
	Coating cells interior with dyes	Staining cell interior with dyes, increasing laser absorption in any part of cell interior including but not limited to vacuole, cytoplasm, organelles or other parts of cell interior; dye may cause laser to fail to penetrate cell interior deeply, leading to cell protection from damage or other effects leading to increased cell survival or increase macromolecule uptake
	Coating tonoplast/vacuole with dye	Delivering dyes into the vacuole and/or the tonoplast (vacuole membrane) to block laser irradiation from penetrating deeply into cells, leading to cell protection from damage or other effects leading to increased cell survival or increase macromolecule uptake
Altered physical conditions	Additives that increase stability of molecule to be delivered	Adding chemicals or compounds to the cell suspension or the solution of the molecule to be delivered into the cells, or both, to increase molecule stability in the presence of the cells, or in the course of laser irradiation, or in transport through the cell to its target location
	Additives that increase the rate of molecule transport to the correct cellular compartment	Adding chemicals or compounds to the cell suspension or the solution of the molecule to be delivered into the cells, or both, to increase the rate or frequency of transport of the molecule to the correct compartment within the cell (i.e. DNA delivery into cell nuclei); examples are salts that interact with DNA and facilitate their passage in(to) the cell, DNA-binding peptides containing a nuclear localization signal, DNA condensation agents, polyamines, cationic peptides that bind DNA, etc.
	Controlled pressure irradiation	Changing air or water pressure around cells or inside containment vessel before and/or during and/or after irradiation; exposing the cells or tissues to pressure higher or lower than atmospheric pressure before and/or during and/or after irradiation
Cell treatments	Controlled temperature irradiation	Manipulating temperature of tissue and/or suspension solution and/or containment vessel before and/or during and/or after irradiation
	Irradiation in high- or low-osmolality media	Use of media with higher or lower than typical osmolality to manipulate cell size, cell soluble content, cell turgor pressure or any aspect of cell properties that influence delivery of molecules into cell
	Irradiation with sonication	Treatment of cells with audible or ultrasonic frequencies before, during or after laser irradiation
	Agents or treatments affecting cell walls	Softening cell walls or partially digesting cell walls with chemical or enzymatic treatments; treatment of cells or tissue to be irradiated with any chemical or biological agents that alter cell wall structure, thickness, pore size, strength, elasticity or any other properties
	Agents or treatments affecting cell membranes	Treatment of cells or tissue to be irradiated with any chemical or biological agent that alters membrane composition, thickness, fluidity, porosity, permeability, rate of turnover or any other properties; affected membranes can be plasma membrane, or any organelle membrane or other intracellular membrane, examples are detergents (for permeabilizing membranes, surfactants, ionophores, pore-forming peptides
Cell treatments	Agents or treatments affecting organelles, cell structure	Treatment of cells or tissue to be irradiated with any chemical or biological agent that alters size, structure or properties of cellular organelles such as vacuoles, Golgi apparatus, ER, nucleus, with specific emphasis on changes that affect the transport of molecule within the cell or changes that affect the rate or frequency of molecule uptake into the nucleus

[0062] Table 2 lists examples of chemicals that can have effects on plant cell membranes, cell walls and vacuoles, including several ionophores.

Table 2: Compounds affecting plant cell vacuoles, cell walls, membranes etc

Compound	Activity
3-methyladenine	inhibitor of autophagy in animal and plant cells
Sodium orthovanadate	ATPase inhibitor
Concanamycin A	specific vacuolar H ⁺ -ATPase inhibitor
Bafilomycin A1	macrolide antibiotic, specific inhibitor of vacuolar-type H ⁺ -ATPase
Fumonisin B1	inhibitor of ceramide biosynthesis, disrupts vacuolar membranes
Monensin	Na ⁺ ionophore
SQL-Pr	synthetic Na ⁺ ionophore
Nigericin	K ⁺ ionophore
Calimycin (A23187 free acid)	ionophore for divalent cations
Streptomyces chartreusensis	
4-Bromo-A23187	ionophore for divalent cations
CA 1001	Ca ²⁺ ionophore, highly selective
Nystatin	non-specific aition ionophore with preference for Na ⁺
Valinomycin, Streptomyces fulvissimus	K ⁺ ionophore that also transports oher monovalent cations
Gramicidin A	Channel-forming pentadecapeptide, ion channel for monovalent cations
Ionomycin, Free Acid	Divalent cation-specific ionophore
Salinomycin	Polyether antibiotic, K ⁺ ionophore
Palytoxin	Divalent cation-specific ionophore, inhibitor of K ⁺ /Na ⁺ ATPases
Beauvericin	depsipeptide antibiotic capable of transporting mono- and divalent cations
Butylated hydroxytoluene (BHT)	antioxidant; causes organelle changes including vacuole shape
Filipin III	ergosterol ligand, inhibits vesicle fusion
Nystatin	ergosterol ligand, inhibits vesicle fusion
Amphotericin B	ergosterol ligand, inhibits vesicle fusion
Fenpropimorph	sterol biosynthesis inhibitor, sold as pesticide
Fusicoccin	increases cell wall plasticity, fungal toxin
Trehalose	cryoprotective agent, protects against stresses
Raffinose	cryoprotective agent, protects against stresses
Glycinebetaine	cryoprotective agent, protects against stresses

[0063] The following examples are offered to illustrate, but not limit, the invention.

The laser parameters used in the examples are generally as follows: experiments were performed with a Raydiance Inc. prototype ultra-short pulse (USP) laser, typically with the following pulse parameters: full width at half maximum (FWHM) of 1.08 ps, minimum percent energy of 99.82% in a 10 ps interval spanning the peak of the pulse, minimum percent energy of 100% in a 20 ps interval spanning the peak of the pulse, frequency range of 10 Hz to 500 kHz, and power range of 0 to 5 μ J.

Example 1

Use of hydrogel and gold particles with broad-beam irradiation for DNA delivery into plant cells

[0064] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed twice with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells resuspended in the same solution, inverted gently.

[0065] 2 ml aliquots of solid medium consisting of 3% sucrose, 0.5 g/L MES, 0.26% Gelrite, 2 mg/ml 2,4 dichlorophenoxyacetic acid, pH 5.8 were added to 50 mm diameter petri dishes pretreated with the wetting agent Silwet L-77 and were allowed to solidify.

[0066] Spermidine and plasmid DNA encoding a red fluorescent protein gene were mixed and added to the washed callus at final concentrations of 20 mM and 10 ng/μl, respectively. The callus was incubated for 5 minutes. 250 μg of 1 μm gold particles were spotted on to the gel layer with a pipette. The callus was removed from the DNA/spermidine solution and placed on top of the gold particles/gel layer. 1ml of the same 10 ng/μl DNA solution was then added to the dish on top of the cells.

[0067] A 1552 nm USP laser was focused into the gel layer with a Mitutoyo M Plan/Apo (NIR 20X, NA = 0.40) objective. The gel layer was irradiated with a power and frequency setting of 5μJ, 500 kHz and 10 psec pulse duration. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the gel, gold particles, and callus/DNA. Movement was at 1 mm/sec in a gridded switchback pattern covering an area of approximately 1 cm² with 1 mm spacing between tracks.

[0068] The irradiated callus was then placed back on a fresh plate of callus medium and allowed to grow at room temperature for 48 hours. Detection of cells expressing the red fluorescent protein was carried out using a Zeiss Discovery V12 Stereomicroscope with fluorescence capability. FIG. 1 provides two images of cells with fluorescent nuclei indicative of successful DNA delivery into plant cells using the gel/particle approach with broadbeam laser irradiation.

Example 2

Use of a Na⁺ ionophore to enhance USP laser irradiation for DNA delivery into plant cells

[0069] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed twice with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells resuspended in the same solution, inverted gently.

[0070] Stock 1M or 500 mM monensin solutions were diluted in 3% sucrose, 20 mM KCl, 10 mM MES, pH 6. Washed callus cells were added to these solutions and incubated for one hour at room temperature. Different samples were placed into monensin at staggered times according to their order and time of irradiation.

[0071] Individual callus pieces were removed from the incubation solution at the one hour time point and submerged in 3% sucrose, 20 mM KCl, 10 mM MES, 20 mM spermidine (irradiation solution). The callus pieces were then transferred to a glass bottom plate with 250 µl fresh irradiation solution containing 10 ng/µl plasmid DNA encoding the firefly luciferase gene.

[0072] A 1552 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Mitutoyo M Plan/Apo (NIR 20X, NA = 0.40) objective. The cells were irradiated with settings of 0.5-5 µJ, 500 kHz and 10 psec pulse duration, using packets of 1-10 laser pulses separated by 32,768 pulse equivalents without irradiation. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Movement was at 1 mm/sec in a gridded switchback pattern covering an area of approx. 1 cm² with 1 mm spacing between tracks.

[0073] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 2 shows the measure of fluorescence (relative luciferase units) for 7 test samples as compared to 3 control samples (no DNA, no additive/monensin and no laser controls). All test samples show luciferase activity above background indicative of successful DNA delivery.

Example 3

Optimizing the use of a Na⁺ ionophore and UV wavelength to enhance USP laser irradiation for DNA delivery into plant cells

[0074] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells resuspended in the same solution, inverted gently.

[0075] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 µl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/µl plasmid DNA encoding the firefly luciferase gene) and varying concentrations of monensin from 5-1000 µM.

[0076] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W pulse running at 50 kHz with a 10 psec pulse duration. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Velocity of stage motion was 1 mm/sec. Movement was in a gridded switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0077] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 3 shows relative luciferase activity for 9 test samples as compared to 3 control samples (no DNA control 1, no additive/monensin control 2 and no laser control 3). All test samples show luciferase activity above background indicative of successful DNA delivery. A monensin concentration of 70 uM shows the best result indicating an optimal effective range between 50 and 100uM.

Example 4

Optimizing the use of a Na⁺ ionophore using different incubation times to enhance USP laser irradiation for DNA delivery into plant cells

[0078] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells were resuspended in the same solution plus or minus detergents, inverting gently.

[0079] Ionophores were added to the incubation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) at 50uM final concentration. Samples were incubated for 5, 30 or 60 minutes and then callus was removed and washed in 3 % sucrose, 20 mM KCl, 10 mM MES, pH 6. Flushing of samples was done in petri dish with samples submerged in wash solution for one minute on a rocking table. Cells were then placed in irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/μl plasmid DNA encoding the firefly luciferase gene).

[0080] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 μl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/μl plasmid DNA encoding the firefly luciferase gene). In addition to the standard resuspension solution, monensin was added and incubations were carried out as shown in FIG. 4. Samples 1 and 2 both received washes in standard resuspension buffer while samples 3, 4, and 5 did not.

[0081] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W pulse, a frequency of 10Hz and a 10 psec pulse duration. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Movement was at 1 mm/sec in a gridded switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0082] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 4 shows relative luciferase activity for the five experimental conditions as compared to two controls (no monensin control 1 and no laser control 2). Samples three, four, and five show luciferase activity above background indicative of successful DNA delivery. These successful conditions demonstrate that washing is beneficial post irradiation following incubation in monensin, and that cell incubation time in the solution containing monensin can range from 5 to 60 minutes.

Example 5

Optimizing power and packet dosage to enhance USP laser irradiation for DNA delivery into plant cells

[0083] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells were resuspended in the same solution, inverting gently.

[0084] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 µl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/µl plasmid DNA encoding the firefly luciferase gene).

[0085] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated at various powers and various packets with a frequency of 50 kHz and a 10 psec pulse duration. Power was adjusted between 0.3 and 0.4 W and packets at one, two, and ten pulses per packet. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Movement was at 1 mm/sec in a gridded switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0086] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 5 shows relative luciferase activity for eight experimental conditions as compared to two controls (no monensin control 1 and no laser control 2). Samples one, four, and five show luciferase activity well above background indicative of successful DNA delivery. Power levels of 0.4 W were most successful.

Example 6

Optimizing surfactants/detergents to enhance USP laser irradiation for DNA delivery into plant cells

[0087] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3% sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20°C.

[0088] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 µl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/µl plasmid DNA encoding the firefly luciferase gene). This solution also contained a detergent at a final concentration as listed in FIG. 6.

[0089] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W pulse and a frequency of 10Hz, 10 psec pulse duration. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Movement was at 1 mm/sec in a gridded switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0090] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas

luminometer and Promega luciferase assay substrate. FIG. 6 shows relative luciferase activity for eight experimental conditions as compared to two controls (no laser control 1 and no DNA control 2; both of which were detergent-free). Sample 1 and 2 contained Tween 20, Samples 3 and 4 contained Silwet L-77, Samples 5 and 6 contained Pluronic F-68 and samples 7 and 8 contained Triton-X 100 at the indicated concentrations. The odd numbered samples contain the detergents at 0.1% and the even ones at 0.01% as depicted in FIG. 6. Samples one, four, seven, and eight show luciferase activity above background indicative of successful DNA delivery. Triton-X 100 samples performed the best in this case at both concentrations, with the 0.1% concentration being best.

Example 7

Optimizing different speeds of irradiation to enhance USP laser irradiation for DNA delivery into plant cells

[0091] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells were resuspended in the same solution, inverting gently.

[0092] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 µl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/µl plasmid DNA encoding the firefly luciferase gene) and 0.1% Triton-X 100.

[0093] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W power setting at the indicated pulse frequencies and a 10 psec pulse duration. Frequency and velocity of stage motion were varied in a manner that the spacing between pulses on the sample remained the same between experimental conditions and only the speed of stage translation changed. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Laser movement across the sample described a gridded

switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0094] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 7 shows relative luciferase activity for four experimental conditions (samples 1-4) reproduced in samples 5-8 where equivalent conditions show highly reproducible luciferase activities, as compared to two controls (no DNA control 1 and no laser control 2). Sample 2 and the corresponding replicate, sample 6 showed the best results. All test samples show luciferase activity above background indicative of successful DNA delivery. Samples 2 and 6 suggest that the speed at which samples are moving during irradiation may be important and that under the above conditions a speed of 0.5 mm/s performed best. Although theoretically all samples received exactly the same laser dosage and number of pulses, the overall duration of irradiation also varied and may have affected DNA delivery.

Example 8

Optimizing surfactants/detergents to enhance USP laser irradiation for DNA delivery into plant cells

[0095] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells were resuspended in the same solution, inverting gently.

[0096] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) and transferred to a glass bottom plate with 250 µl fresh irradiation/detergent solution containing 10 ng/µl plasmid DNA encoding the firefly luciferase gene.

[0097] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W pulse. Frequency and velocity of stage motion were varied

between 50 - 500 kHz, and 0.1 – 1 mm/sec respectively 500 kHz, with a 10 psec pulse duration in all cases. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. Movement was in a gridded switchback pattern covering an area of approx. 1 cm² with 0.5 mm spacing between tracks.

[0098] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 8 shows relative luciferase activity for five experimental conditions with differing detergents as compared to two controls (no DNA control 1 and no laser control 2). All five detergents showed positive results out of a larger number of detergents and surfactants tested.

Example 9

Optimizing surfactants/detergents together with ionophores to enhance USP laser irradiation for DNA delivery into plant cells

[0099] Soybean callus was grown on 3% sucrose, 0.5 g/L MES, 4.6 g/L MS basal medium 4x Gamborg's vitamins, 0.26% Gelrite, 2 mg/ml 2,4 D, pH 5.8 (callus medium). Whole calli were removed from the medium and washed once with a solution of 3 % sucrose, 20 mM KCl, 10 mM MES, 0.5 mM EDTA pH 6 and then by another wash in the same solution minus EDTA. For each wash, the cells were inverted several times and centrifuged for 7 minutes at 1000 rpm at 20 °C. The wash solution was removed and cells were resuspended in the same solution, inverting gently.

[00100] Individual callus pieces were removed from the resuspension solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6) transferred to a glass bottom plate with 250 µl fresh irradiation solution (3% sucrose, 10 mM MES, 20 mM KCl, pH 6 plus 10 ng/µl plasmid DNA encoding the firefly luciferase gene) and 0.25% Triton X 100.

[00101] A 388 nm USP laser was focused onto the interface between the cells and the glass bottoms of the plates containing the cells with a Thorlabs LMU-20X-NUV Acromatic MicroSpot Focusing Objective (20X, 325 - 500 nm, NA=0.40). The cells were irradiated with a 0.5 W power setting set to 50 kHz with a 10 psec pulse duration. During irradiation, the cells were moved through the path of the laser using a mobile stage that held the plate containing the callus and DNA. The velocity of stage motion was 1 mm/sec. Movement was at 1 mm/sec in a gridded switchback pattern covering an

area of approx. 1 cm² with 0.5 mm spacing between tracks. Ionophores tested are abbreviated in FIG. 9 and were as follows: amphotericin B (Amp), fumonisin B1 (Fus), A 23187, ionomycin, glycinebetaine (Glyc.bet), monensin (Mon), nigericin (Nig), butylated hydroxytoluene (BHT), filipin III, nystatin, 3-methyladenine (3-met), sodium orthovanadate (Sod.Orth), concanamycin A (Concan.), bafilomycin A1 (Bafilomy.), and fumonisin B1 (Fumon.).

[00102] Irradiated calli were placed back on fresh callus medium and incubated at room temperature for 48 hours. Cells were disrupted by manual grinding and cell extracts were assayed for luciferase activity with a Turner Biosystems Veritas luminometer and Promega luciferase assay substrate. FIG. 9 shows relative luciferase activity for fifteen experimental conditions as compared to two controls (no DNA control 1 and no laser control 2). All test samples show luciferase activity above background indicative of successful DNA delivery. Monensin and concanamycin showed the best results.

[00103] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method for introducing macromolecules into a cell having a cell wall, said method comprising:
 - (i) contacting a plurality of cells having cell walls with a macromolecule-containing solution;
 - (ii) exposing said plurality of cells to ultraviolet laser beam pulses of less than 1 nanosecond generated by an ultra-short pulse laser; and
 - (iii) allowing said plurality of cells to maintain viability and/or grow under conditions that permit detection of the macromolecule.
2. The method according to claim 1, wherein said cell is selected from the group consisting of a bacterial cell, an archaeal cell, a fungal cell, a yeast cell, an algal cell, and a plant cell.
3. The method according to claim 2, wherein said plant cell being contacted is present in a sample of plant tissue.
4. The method according to claim 3, wherein said plant tissue is selected from the group consisting of leaf disks, petioles, roots, bulbs, tubers, hypocotyls, shoots, meristematic tissues, inflorescences, reproductive cells, pollen, microspores, embryos, calli, fruit, seeds and parts of any thereof.
5. The method according to claim 1, wherein said macromolecule-containing solution contains a plurality of macromolecules selected from the group consisting of a protein, a peptide, an amino acid, a nucleic acid, DNA, RNA, an oligonucleotide, a lipid, a carbohydrate, and a combination of any thereof.
6. The method according to claim 1, wherein said macromolecule-containing solution contains a plurality of macromolecules selected from the group consisting of histones, transcription factors, nucleolamins, RNA-binding proteins, DNA-binding proteins, DNA-replication proteins, DNA recombination proteins and DNA-repair proteins.

7. The method according to claim 1, wherein said ultraviolet laser beam pulses have a wavelength of less than 400 nm
8. The method according to claim 7, wherein said cell is a plant cell and said macromolecule-containing solution comprises nucleic acid molecules.
9. The method according to claim 1, wherein said plurality of cells is placed into a translucent container, wherein said container permits transmission of said ultraviolet laser beam pulses through a surface of said container.
10. The method according to claim 9, wherein said ultraviolet laser beam pulses are focused onto an interface between said plurality of cells and a surface of said container.
11. The method according to claim 1, wherein said exposing step (ii) comprises moving said plurality of cells through a path of said ultraviolet laser beam using a mobile support upon which a container containing said plurality of cells is positioned.
12. The method according to claim 1, wherein said exposing step (ii) comprises moving said ultraviolet laser beam across said plurality of cells which remain in a fixed position.
13. The method according to claim 1, wherein said exposing step (ii) comprises physically agitating said plurality of cells simultaneously with delivering said ultraviolet laser beam pulses.
14. The method according to claim 11, wherein said movement is at about 0.1 - 100 mm/s in a gridded pattern covering an area of about 0.1 - 10 cm².
15. The method according to claim 1, wherein said ultraviolet laser beam is about 2-50 μ m in spot diameter.
16. The method according to claim 1, wherein said ultraviolet laser beam is less than 2 μ m in spot diameter.

17. The method according to claim 1, wherein said ultraviolet laser beam is 0.1-1 μm in spot diameter.
18. The method according to claim 5, wherein said macromolecule-containing solution further comprises an additive.
19. The method according to claim 18, wherein said additive is selected from the group consisting of a polyamine, a DNA condensation agent, a DNA-binding dye, and a combination of any thereof.
20. The method according to claim 19, wherein said polyamine is spermidine or spermine.
21. The method according to claim 19, wherein said DNA condensation agent is hexamine cobalt.
22. The method according to claim 19, wherein said DNA-binding dye is selected from the group consisting of bis-benzamide, Hoechst 33258 and Hoechst 33342, and ethidium bromide.
23. The method according to claim 18, wherein said additive is selected from the group consisting of a poly-lysine peptide, a poly-arginine peptide, a DNA-binding protein, a peptide containing a nuclear localization signal, polyethylene glycol, dextran sulfate, polyvinylpyrrolidone, and a combination of any thereof.
24. The method according to claim 18, wherein said additive is an ionophore.
25. The method according to claim 24, wherein said ionophore is monensin.
26. The method according to claim 18, wherein said additive is a detergent or a surfactant.
27. The method according to claim 18, wherein said additive is a combination of an ionophore and a detergent or surfactant.
28. The method according to claim 1, wherein said plurality of cells is subjected to a pretreatment prior to contacting step (i).

29. The method according to claim 28, wherein said pretreatment comprises contacting said plurality of cells with an ionophore and/or a detergent or surfactant.

30. The method according to claim 1, wherein said exposing step (ii) comprises a continuous movement of said ultraviolet laser beam across said plurality of cells by moving said ultraviolet laser beam and/or said plurality of cells.

31. The method according to claim 1, wherein said exposing step (ii) comprises intermittently stopping relative movement between said ultraviolet laser beam and said plurality of cells by stopping movement of said ultraviolet laser beam and/or said plurality of cells to turn on and/or off said ultraviolet laser beam pulses; to alter pulse number, frequency, duration, focal plane, or strength of said ultraviolet laser beam pulses; or to adjust packets of pulses for any parameters.

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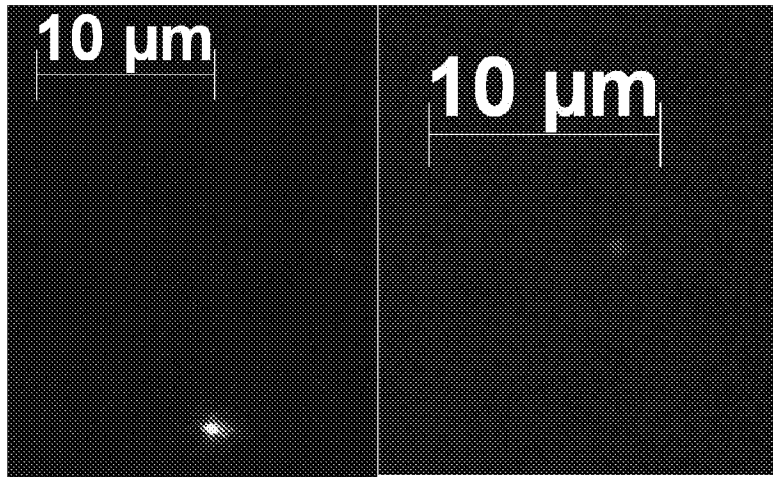


FIG. 1

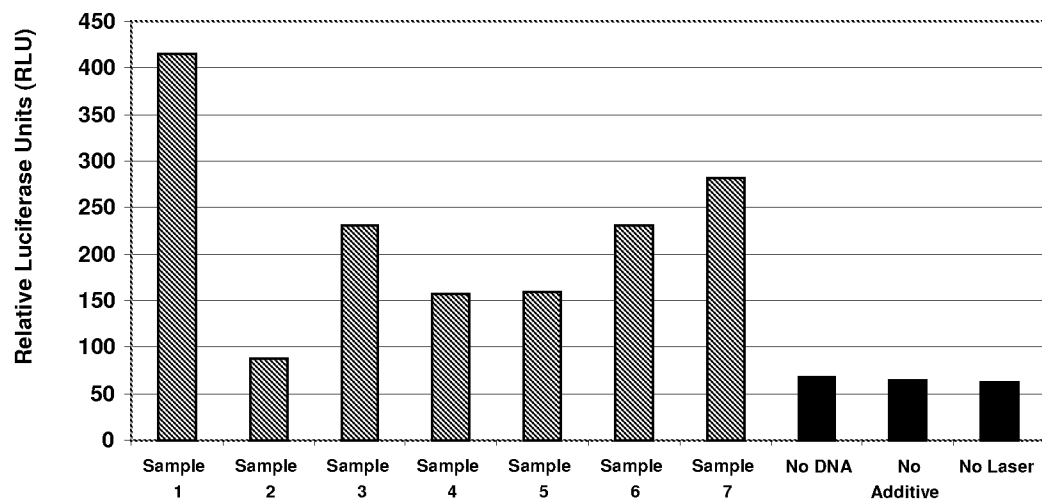
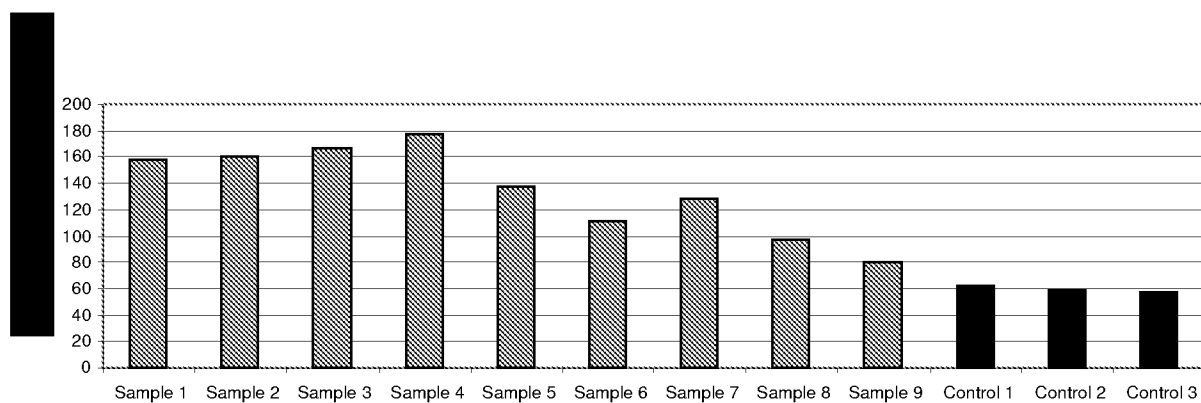
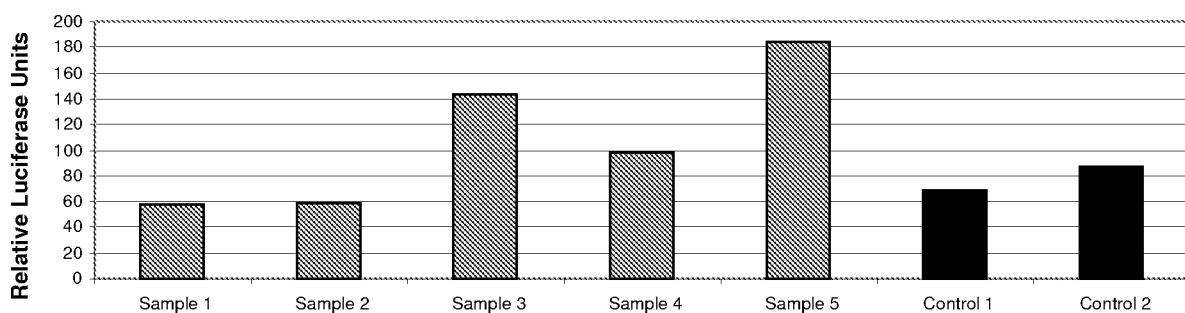


FIG. 2

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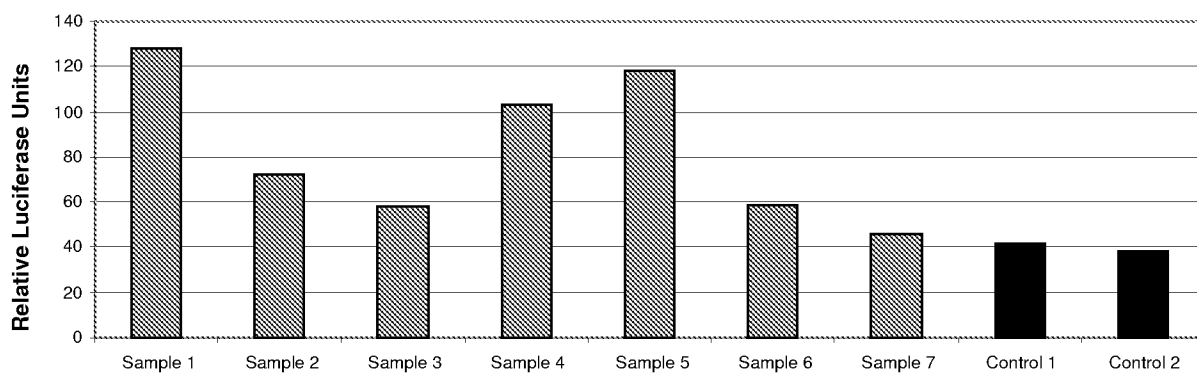
Sample	1	2	3	4	5	6	7	8	9	Control 1	Control 2	Control 3
Concentration (uM)	5	25	50	75	100	150	200	500	1000	50	0	50

FIG. 3

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Control 1	Control 2
Incubation Time (min)	30	60	5	30	60	0	0
Post Incubation wash	no	no	yes	yes	yes	no	no

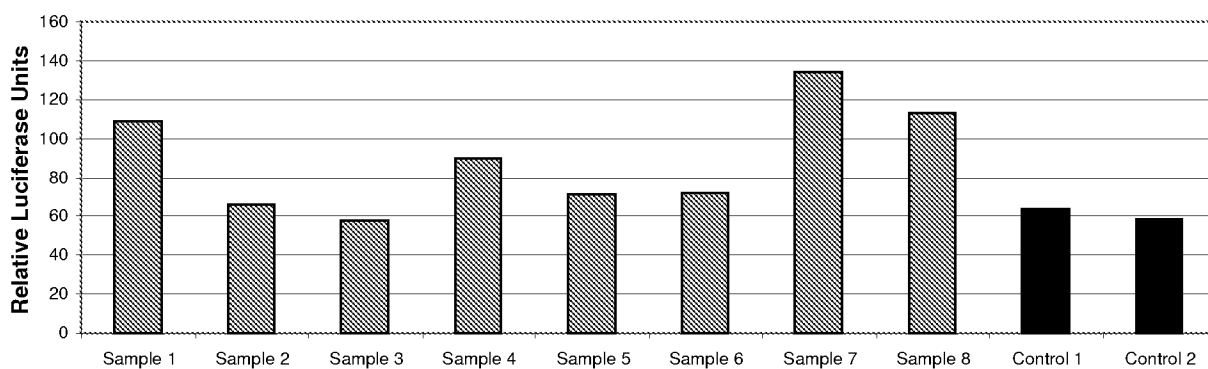
FIG. 4

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	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Control 1	Control 2
Power (W)	0.4	0.3	0.4	0.3	0.4	0.3	0.5	0.5	0
Packets	1	1	2	2	10	10	1	10	0

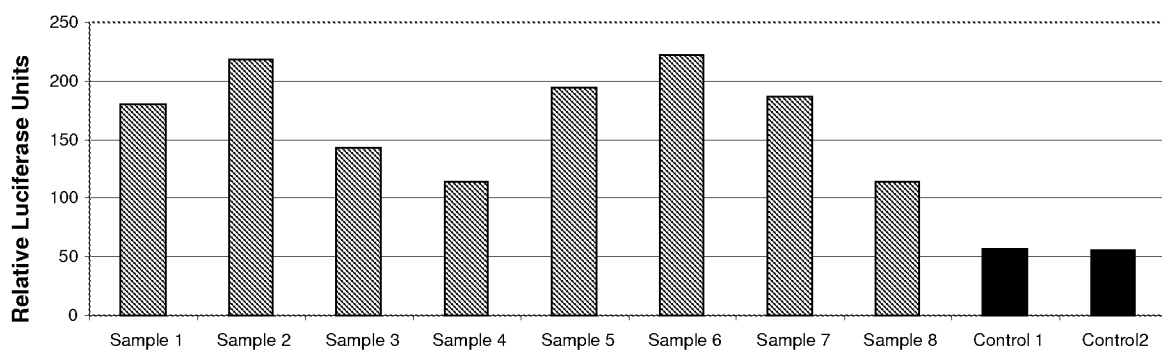
FIG. 5



	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Control 1	Control 2
Detergent/Surfactant	Tween	Tween	Silwet	Silwet	Pluronic	Pluronic	Triton-X	Triton-X	Triton-X	Triton-X
Concentration (%)	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0	0

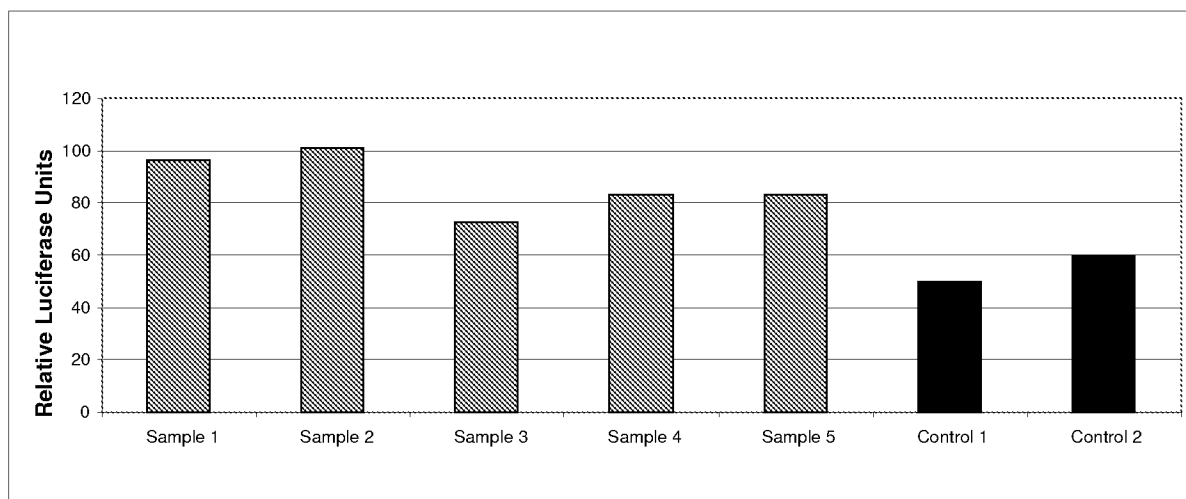
FIG. 6

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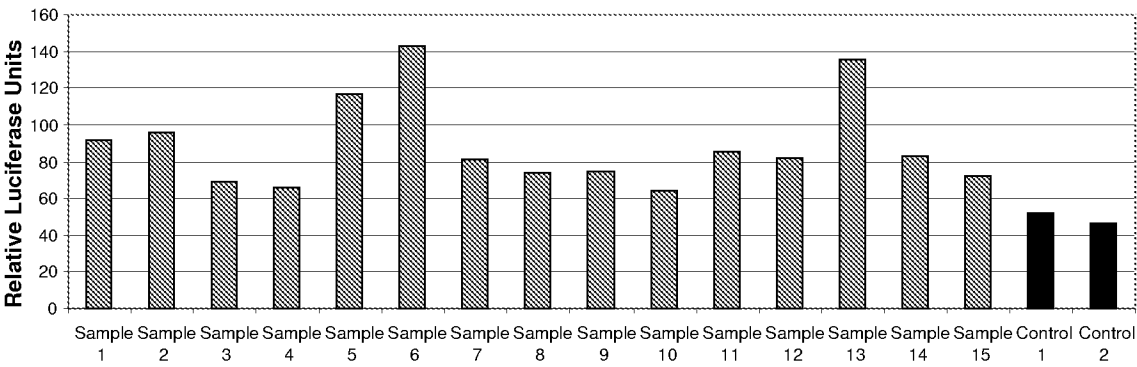
Sample	1	2	3	4	5	6	7	8	Control 1	Control 2
Freq (Hz)	500,000	250,000	100,000	50,000	500,000	250,000	100,000	50,000	10	0
Speed (mm/s)	1	0.5	0.2	0.1	1	0.5	0.2	0.1	1	1

FIG. 7



Sample	1	2	3	4	5	Control 1	Control 2
Detergent/Surfactant	Sodium deoxycholate	Brij 35	Pluronic F-68	Triton X-100	Tween 20	0	0

FIG. 8



Sample	1	2	3	4	5	6	7	8	9
Inophore	Amp	Fus	A 213187	Ionomycin	Glyc.bet	Mon	Nig	BHT	Filipin
Sample	10	11	12	13	14	15	Control 1	Control 2	
Inophore	Nystatin	3-Met	Sod.Orth	Concan.	Bafilomy.	Fumon.	no DNA	No Laser	

FIG. 9