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Selective Gene Transfection of Individual Cells *In Vitro* with Plasmonic Nanobubbles

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Abstract

Gene delivery and transfection of eukaryotic cells is widely used for research and for developing gene cell therapy. However, the existing methods lack selectivity, efficacy and safety when heterogeneous cell systems must be treated. We report a new method that employs plasmonic nanobubbles (PNBs) for delivery and transfection. A PNB is a novel, tunable cellular agent with a dual mechanical and optical action due to the formation of the vapor nanobubble around a transiently heated gold nanoparticle upon its exposure to a laser pulse. PNBs enabled the mechanical injection of the extracellular cDNA plasmid into the cytoplasm of individual target living cells, cultured leukemia cells and human CD34+CD117+ stem cells and expression of a green fluorescent protein (GFP) in those cells. PNB generation and lifetime correlated with the expression of green fluorescent protein in PNB-treated cells. Optical scattering by PNBs additionally provided the detection of the target cells and the guidance of cDNA injection at single cell level. In both cell models PNBs demonstrated a gene transfection effect in a single pulse treatment with high selectivity, efficacy and safety. Thus, PNBs provided targeted gene delivery at the single cell level in a single pulse procedure that can be used for safe and effective gene therapy.

Delivery of specific molecules into target cells is a basis for gene cell therapies, biotechnologies and agricultural technologies. This process can be achieved with various methods of gene transfection, transduction, cell poration or injection [1–3]. Regardless of the end effect (transfection or injection), all of these methods share one common procedure, that is the use of intracellular delivery of genes or other molecules without damaging either the cell or the cargo. The ideal delivery and transfection methods should have high transfection efficiency, low toxicity and single cell selectivity of target cells, while also being able to treat simultaneously heterogeneous systems with many different cells.

Current methods include three major groups: viral (transduction) [4–7], chemical [8–11] and physical (including direct micro injection [1,12]). The major drawbacks of virus-mediated transduction are immunogenicity and cytotoxicity, an inflammatory reaction and an

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insertional mutation. Another disadvantage of this method is that a virus package has limited space for a foreign gene to keep infectivity. For these reasons, much effort has been made to develop non-viral transfection methods. Chemical transfection methods [8–11] are also widely used in research and were the first to introduce foreign genes into mammalian cells [11]. The transfection efficacy of chemical methods is largely dependent on factors such as nucleic acid/chemical ratio, solution pH, cell type and membrane conditions. Consequently, these methods are unstable and insufficient. The physical IMT methods represent the latest and the widest generation of techniques that include biolistic particle delivery [13–15], electroporation [16–19], sonoporation [20,21] and optical transfection [22–42]. Biolistic particle delivery is limited by shallow penetration of DNA into the tissue, and physical damage to samples [13–15]. The efficacy of electroporation (the exposure of living cells to short and intense electric pulses) requires the optimization of protocols for each specific type of cell [16–19] and is associated with high toxicity and low selectivity. Sonoporation (the application of ultrasound to permeabilize cell membranes in order to improve the internalization of large molecules), causes the breakdown of the cell cytoskeleton and also has low selectivity [20,21]. Micro-injection methods may provide single cell selectivity but require precise manipulations with individual cells and cannot treat thousands of cells simultaneously, especially in heterogeneous cell systems such as blood or bone marrow [1,12].

Laser-enhanced methods have a quarter of a century of history [24] and employ several laser-induced processes: heating [25], shockwave generation [28–31] and plasma formation during optical breakdown [22,23,28]. Femtosecond laser pulse-induced optical breakdown was shown to be a very precise tool for non-invasive transfection of individual cells [22,23,33,35], however, this method cannot be applied simultaneously to many cells and requires the precise positioning of the cell and the laser beam. Other optical methods were applied *in vivo* [34,43] although required focusing the laser beam into a single cell. Additionally, all the aforementioned optical methods lack selectivity, efficacy and safety and this limits their successful translation into clinical applications. The aforementioned limitations of optical method of gene delivery and transfection can be alleviated by using cell-specific micro and nanoparticles [39–42,44,45]. Such a combination allows a decrease in optical intensities and the associated radiation loads. Plasmonic nanoparticles targeted to specific cells were used as local converters of light to heat and their photothermal effect was shown to correlate to gene transfection [39,40]. However, no specific physical mechanism of such transfection was verified so far, partly because of the failure of direct observation and the characterization of an active transfection-driving physical phenomena in specific individual cells. In summary, the current challenge of gene transfection methods can be resumed as how to deliver molecules to individual specific cells (including adherent and suspension, *in vitro* and *in vivo*) among background cells, with high efficacy, low toxicity and high throughput (i.e. the treatment of many cells)?

We hypothesized that by modifying our recently introduced plasmonic nanobubble (PNB) technology [46–50], we could develop an entirely new delivery and transfection method that would combine single cell selectivity and high efficacy of the simultaneous treatment of many cells in a fast single laser pulse procedure. A PNB is a novel cellular agent with a dual and tunable mechanical and optical action that utilizes the formation of the vapor nanobubble around the plasmonic nanoparticle upon its transient heating with a short laser pulse through the mechanism of plasmon resonance. It produces a localized mechanical, non-thermal impact to the cell [51]. We recently showed that PNBs can be efficiently used at the cellular and tissue level *in vitro* and *in vivo* as diagnostic, therapeutic, delivery and theranostic agents [46–50]. However, PNBs were never before considered for gene delivery and transfection. This experimental work reports the gene transfection of living cells with PNBs *in vitro*.

2. Materials and Methods

2.1. Principle of intracellular delivery of molecular cargo with plasmonic nanobubbles

A PNB emerges when a plasmonic gold nanoparticle (NP) is locally overheated with a short laser pulse. As a result the NP heats and evaporates a thin volume of the surrounding medium, thus creating a vapor nanobubble that expands and then collapses within a short nanosecond. The fast expansion of the vapor bubble has a localized mechanical and non-thermal impact on the environment. The PNB can be precisely controlled through the fluence of the excitation laser pulse [51–53] and by generating PNBs only around NP clusters while producing no PNBs around single NPs or their small clusters. In addition, the vapor-liquid boundaries of the bubbles scatter light very well, thus enabling simultaneous optical sensing of PNBs and associated targets [46,54,55]. After the successful selective and non-invasive generation of PNBs in target cells [48,50], we suggested that small PNBs could transiently open the cellular membrane and create an inbound transient jet that could inject extracellular molecules into individual specific cells without damaging them (Fig. 1).

First of all this method involves the delivery of gold NPs through their selective clusterization in target cells. The selective generation of a PNB requires the formation of at least one relatively large cluster of gold NPs that acts as the PNB source in a target cell. The NP clusters were selectively formed as a result of the antibody-receptor interaction and a follow-up endocytosis of the membrane-accumulated NPs [48,50,53,56]. The NP cluster represents tightly packed 4–20 NPs that are in close contact and thus form a joint thermal field around the cluster upon its exposure to a short laser pulse. It must be pointed out that the molecular cargo is not associated with NPs (as in many other NP-based methods) because laser-induced heating of gold NPs to a high temperature would inevitably destroy any molecules linked with the NPs. Instead, we employ free extracellular molecules that are mechanically (hydrodynamically) injected into the cell through the transient hole in the membrane during the expansion and collapse of the PNB.

The delivery mechanism can be described through the following sequence (Fig. 1):

- selective formation of plasmonic (gold) NP clusters in target cells (Fig. 1a);
- exposure of a tissue or the volume with many cells to a short laser pulse (green arrows at Fig. 1b);
- absorption of light by gold NPs and transient heating of gold NPs above the boiling threshold temperature for the cellular environment;
- generation of vapor nanobubble around NP cluster (a PNB, Fig. 1b);
- mechanical disruption (poration) of the cellular membrane by expanding PNB (Fig. 1b);
- hydrodynamic injection of the extracellular molecules through the transient hole by the hydrodynamic effect, or jet, that emerges during the collapse of the PNB (Fig. 1c).

As a result, extracellular molecules are delivered into the cellular cytoplasm. In addition, optical scattering by the PNB of the probe laser radiation will detect a target cell and will guide the injection process because the mechanical effect of the PNB correlates with its optical scattering [50,51,55]. The safety of PNBs to the molecular cargo was recently verified experimentally for the liposome model where PNBs provided the controlled release of fluorescent proteins from individual liposomes although without any IMT effect [49].

2.2. Cell models

We used two well-characterized *in vitro* models to demonstrate the feasibility of using PNBs for gene transfection since the clinical application of gene transfection (cell gene therapy) employs *ex vivo* treatment of the cells which are then transplanted into the patients. We envision that the *in vitro* models performed in this study may provide the basis for evaluating the potential clinical usage of PNBs as a tool for gene therapy, which is performed exclusively with *ex-vivo* manipulated cells such as T-lymphocytes or hematopoietic stem cells [57,58]. Firstly, Jurkat 32 and JRT3-T3.5 cell lines were used to evaluate the intracellular delivery on DNA-plasmid with PNB. We also used bone marrow (BM) hematopoietic progenitor cells (CD34-positive) and CD34-negative cells for an estimation of the clinical applicability of the suggested method for intracellular delivery.

CD3-positive J32 (test) and CD3-negative JRT3-T3.5 (control) clones of Jurkat T-cell leukemia cell lines were purchased from the American Type Culture Collection. The J32 cells were selectively targeted with gold NPs covalently conjugated with anti-CD3 monoclonal antibody OKT3 (BioAssay Works L.L.C., Ijamsville, MD). Cells were resuspended in a serum free RPMI-1640 medium (Invitrogen, Grand Island, NY) containing OKT3-conjugated NPs at a concentration of 5 μ l NP:1 ml media and incubated with the NP-containing media for 45 minutes at 37°C in CO₂ incubator. Following incubation, the cells were washed to remove free NPs and finally suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Inc., Grand Island, NY) and DNA-plasmid (pHIV-7-GFP [59,60]) with a concentration of 10 μ g/ml, and placed in growth chambers (Lab-Tek II, Thermo Fisher Scientific). Then the cells were treated with laser pulses. After this laser treatment cells were placed in an incubator under standard cultivation conditions. Cell viability after NP-treatment was analyzed using trypan blue staining.

Human primary bone marrow cells were obtained from de-identified leftover marrow samples for evaluating hematologic disorders. The usage of these samples was approved by The Methodist Hospital Research Institute Review Board. The hematopoietic stem cells (CD34-positive cells with varying degrees of CD117 expression) were isolated using magnetic beads conjugated with antibodies against CD34. The stem cells are known to be difficult for gene delivery using currently available methods, and were selected for the further evaluation of our approach. CD117-negative cells within the CD34-positive cells were used as the internal negative control in this model. For the selective targeting of CD117-positive cells we used gold NPs covalently conjugated with monoclonal antibody CD117 (BioAssay Works L.L.C., Ijamsville, MD). Cells were treated with NP-CD117 conjugates according to procedure described above for culture cells, and were placed in the same growth chambers in RPMI-1640 medium with serum and antibiotics and DNA-plasmid (containing GFP and miRNA sequence targeting p38 MAPK) with a concentration of 15 μ g/ml. After PNB-treatment, cells were placed in an incubator under standard cultivation conditions and GFP-fluorescence and cell viability were analyzed at 0, 24 and 48 hours. As a positive control for detecting GFP using our system, we used GFP-positive RPMI 8226 cells which were transduced with GFP plasmid and purified by FACS sorting for GFP-positive cells as described in [61].

2.3. PNB generation and detection

Optical generation and detection of the PNBs was performed with a photothermal laser microscope that we developed previously [51,54]. In our work we employed a pulse of length 0.5 ns, 532 nm (laser model STA-01 SH, Standa Ltd, Vilnius) for PNB generation. The pump laser beam was directed into the illumination path of an inverted optical

microscope and was focused into the sample. Single cells were studied. Imaging of the PNB was realized in two simultaneous modes: time-resolved imaging and time response. Time-resolved imaging was realized by using side illumination of the sample with a pulsed probe laser beam at a wavelength of (690 nm) with a 10 ns delay after pump laser pulse (532 nm). Time-resolved images were registered by a CCD image detector (Luca DL-658M, Andor Technology, Ireland). For measuring the PNB lifetime, an additional continuous probing beam (633 nm) was directed at the sample and focused on it collinearly with pumping laser beams, and its axial intensity was monitored by a high-speed photodetector (PDA110AC, Thorlabs Inc.) and 400-MHz LeCroy Wavesurfer oscilloscope. The PNB-induced scattering of a part of the probing beam decreased its axial amplitude resulting in a dip-shaped output signal. Each single cell was placed in the center of laser beams and irradiated with a single pump laser pulse. The images and time responses from each cell were obtained simultaneously. All cells were irradiated in consecutive order in chambers and the PNBs quantified by measuring their lifetime (size) and bubble generation probability at specific laser pulse fluence. The fluence of the laser pulse is defined as the optical energy per unit of square. This parameter is the most important for a quantitative description of the PNB, because the photothermal effect of plasmonic (gold) NPs depends upon the amount of optical energy that was absorbed by the NPs [62,63]. A short and fixed duration of the laser pulse (0.5 ns) and the pulse fluence fully characterized pulsed irradiation.

2.4. GFP imaging

Bright field and fluorescent imaging of individual living cells was realized with the same experimental set up as that described above. Fluorescent images were obtained for each individual cell at 0, 24 and 48 hours after PNB generation. We used an additional 473 nm continuous laser for the excitation of GFP without removing the cells from the culture wells. The pixel image amplitude of the GFP-specific fluorescence was measured for individual cells. The cells were considered as GFP-positive when the amplitude of GFP fluorescence exceeded the level of such fluorescence obtained for a positive control (the cells stably transfected with GFP). The transfection efficacy was defined as the ratio of the number of GFP-positive cells to the number of PNB-positive cells. Since the GFP fluorescence and PNB generation were studied in individual cells, this experimental method provided a highly precise analysis.

2.5. Control methods

2.5.1—The coupling of the receptor-specific antibodies to the cell membranes was verified with flow cytometry using standard methods as described previously [61].

2.5.2—Cell viability was measured with the trypan blue extinction method by counting the level of trypan blue-negative cells. The cell viability index was determined as the number of viable test cells divided by the number of viable intact cells. The latter were not exposed to any treatments and were used only for the purpose of reference and for monitoring the viability of the specific cell sample.

2.5.3—The accumulation of gold NPs in living cells was directly monitored with optical scattering imaging performed with a laser scanning confocal microscope LSM-710 (Carl Zeiss MicroImaging GmbH, Germany) in the reflectance mode under excitation with a 633 nm continuous laser. We used the optical scattering by gold NPs for the direct detection of NP clusters in living cells.

3. Results

3.1. NP internalization in cells

CD3-positive J32 and CD3-negative JRT3 clones of Jurkat cell line were treated with OKT3-conjugated gold NPs, and the accumulation of the gold NP conjugates in the cultured J32 and JRT3 cells was imaged with the confocal scattering imaging. We found (Fig. 2b, c, f, g) that (1) NP clusters were formed after 30 minutes of cell incubation with the conjugates, (2) such clusters were located mainly in the cytoplasm near the cellular membrane and (3) some NPs were internalized also by non-target (control) cells (Fig. 2f, g). Despite the non-specific accumulation of gold NPs in the control cells (according to numerous studies and to our previous experimental experience, this effect is unfortunately unavoidable), we observed the formation of the largest NP clusters only in the target cells. In scattering imaging of the object with sub-wavelength size, their image size does not correspond to their real size. However, the amplitude of the scattering image correlates to the size of the scattering object. We used this property of optical scattering to compare the NP clusters in cells (Fig. 2d, h): the target cells yielded much higher scattering amplitudes and, therefore, we concluded that the target cells formed the largest clusters of gold NPs. In all cases we observed the scattering objects very close to the membrane. In particular, Fig. 2c shows the overlay of the bright field and the scattering images with an impression that the scattering objects were inside the cell but this is only projection, the vertical scans of the imaging plane clearly demonstrate that the scattering objects were associated with the cellular membrane. However, the limited resolution of the confocal microscope did not allow us to identify the precise location of NP clusters. Next, we studied plasmonic nanobubbles in individual cells with gold clusters.

3.2. Generation of plasmonic nanobubbles in individual living cells

The exposure of individual living cells to identical single laser pulses (0.5 ns, 532 nm) at a specific fluence level revealed a much bigger difference between target and control cells, both in their PNB images (Fig. 3a, c) and time responses (Fig. 3b, d). According to the PNB images and responses, the PNBs were selectively generated in the target cells.

We varied the laser pulse fluence and measured the PNB generation probability in individual J32 (target) and JRT3 (control) cells (Fig. 4). As can be seen from the graphs, the cluster-bubble mechanism turned out to be much more selective compared to the selectivity of the NP targeting previously discussed. This difference in selectivity was provided by the threshold nature of PNBs: the combination of a relatively low fluence of the laser pulses with the small size of NP clusters in JRT3 cells turned out to be insufficient to generate PNBs.

We used the PNB generation probability to estimate the PNB generation thresholds in the two types of cells: for J32 it was found to be 112 mJ/cm^2 , for JRT3 it was not determined since no PNBs were observed in the range of applied fluencies (Fig. 4a). The next graph (Fig. 4b) showed another useful property of a PNB: its lifetime (that characterizes the maximal size of PNB [51,54] and hence, its IMT effect), correlated with the fluence of the excitation laser pulse, thus allowing the precise control of PNB action in individual cells. We determined the optimal laser fluence for PNB generation in the following transfection experiment. The cells were treated with single laser pulses of 120 mJ/cm^2 .

3.3. cDNA targeting and GFP expression

3.3.1. Cultured cells—The J32 and JRT3 were treated identically with gold NP conjugates (NP-OCT3) and single laser pulses under the presence of extracellular DNA plasmid (pHIV-7-GFP). PNBs were generated and optically monitored in individual cells.

After the PNB treatment, the fluorescent and bright field imaging was performed immediately (0 hour) and after 24 and 48 hours (Fig. 5). In the meantime, the cells were kept in the incubator under standard conditions. The gene transfection effect was monitored through the pixel image amplitude of GFP-specific fluorescence for each individual cell. Those amplitudes were analyzed as a function of time and they were also correlated to the lifetime (that is, the size) of PNBs (Fig. 5g).

We used GFP-positive RPMI 8226 cells as an additional control (Fig. 5e, f) for the reliable identification of the transfection status of the PNB-treated cells. We did not observe any GFP-specific fluorescence in all PNB-treated cells at 0- and 24-hour stages (Fig. 5g). However, after 48 hours, we observed relatively bright GFP-specific fluorescence in J32 PNB-treated cells (Fig. 5a, b and 5g). The control cells exhibited no or very low fluorescence by this time (Fig. 5c, d and 5g). We compared GFP fluorescence (axis Y, Fig. 5g) to the size of PNBs (axis X, Fig. 5g) in individual cells. These two parameters perfectly correlated: no or small PNBs in JRT3 cells were associated with no or very weak fluorescence after 48 hours of cell incubation. To the contrary, the large PNBs in J32 cells were associated with bright fluorescence of GFP (Fig. 5g). Based on the direct detection of PNBs and GFP fluorescence in individual cells, we conclude that the PNBs were responsible for the transfection of GFP.

Next, we analyzed the transfection efficacy and the viability of the NP- and PNB-treated cells (Table 1). These data were obtained through the analysis of individual cells described above in the test and control populations. The efficacy of GFP transfection in target (test) cells achieved 74% (relative to the PNB-positive cells) under the cell viability level of 84%. At the same time the efficacy of the transfection of the control cells was found to be close to zero since the level of their GFP-specific fluorescence did not achieve for the threshold (Fig. 5g) that was used to identify GFP-positive cells. Therefore, the PNB method of intracellular molecular targeting provided high efficacy, selectivity and safety for culture cells. Our next task was to evaluate the method for human cells.

3.3.2. Human stem cells—The suspensions of human stem cells were tested for the expression of specific receptors that were used for targeting gold NPs by flow cytometry using fluorescence-labeled antibodies against CD117. The parameters of PNBs were determined for both cell samples of the test (CD34-positive and CD117-positive cells) and the control (CD34-positive and CD117-negative cells) that were identically targeted with the gold conjugates NP-CD117 antibody. As a result, we optimized the fluence of the laser pulse for the transfection experiments at the level of 150 mJ/cm^2 (Table 2). Also, all PNBs yielded very symmetrical shapes of their time responses that showed no thermal effect after the PNB collapse. This means that single PNBs acted mainly through their mechanical impact and that no direct thermal impact was associated with the PNB action.

The lifetime of PNBs corresponds to their size, and previously we had found PNBs with a short lifetime to be non-invasive for cells [48]. For human cells we were unable to prepare a pure population of CD34-positive CD117-positive cells, though our targets were CD117-positive cells among CD34-positive cells. This complicated the analysis. We addressed this obstacle by obtaining three independent data sets that characterized the level of CD117-positive cells in the population of CD34-positive cells (flow cytometry), PNB generation (optical scattering imaging of PNB and measuring their lifetimes) and analysis of the GFP-specific fluorescence.

Fluorescent imaging of the cells revealed the relatively fast development of the GFP specific fluorescence: it was detected in 24 and in 48 hours. Based on the level of the image amplitude, we discovered the two subpopulations in the test sample (Fig. 6, 7):

1. Sub-population 1 (Fig. 6a) was identified by the cell with bright GFP-specific fluorescence. It counted 10.7% of CD34-positive cells after 24 hours and 9% of the cells in 48 hours. The level of the fluorescent image amplitude is shown in Fig. 7a and in 48 hours it was found to be approximately 13 times higher than the level of the fluorescent image amplitude for the control cells.
2. Sub-population 2 (Fig. 6b) was identified by the cell with dim GFP-specific fluorescence. It counted 66% of CD34-positive cells after 24 hours and 75% of the cells in 48 hours. These signals represent the level of non-specific fluorescence (Fig. 7b).

We noticed that the level of GFP-specific fluorescence in the sub-population 2 was very close to that of the control (CD117-negative cells) and it did not show the dynamics of the sub-population 1. After comparing two independently obtained parameters, the level of CD117-positive cells and the PNB generation probability in the test sample, with the levels of the cells in the sub-populations 1 and 2 (Fig. 7b) we assumed that the PNB generation and the relatively fast dynamics of the GFP-specific fluorescence was associated with the CD117-positive cells in the test sample.

Next, we plotted the fluorescent amplitude as a function of PNB lifetime for the sub-population 1 in the test sample and for the control sample. Again, as in the case with culture cells, we observed a very good correlation of GFP-specific fluorescence with the lifetime of PNBs (Fig. 8). The similarity of the results obtained for culture and human cells suggests one mechanism for inducing the GFP-specific fluorescence, plasmonic nanobubbles.

Finally, we analyzed the efficacy, selectivity and safety of the PNB transfection (Table 3). The selectivity of the transfection for the sub-population 1 was found to be very high: 71% of GFP-positive cells (relative to the PNB-positive cells) in the test sample versus 0% in the control sample. At the same time, the relative viability of the cells was comparable to that of the intact cells. These results were shown not for the whole test sample but only for the sub-population 1 and, therefore, they included an assumption that the subpopulation 2 represented CD117-positive cells. At the time of the experiment we were unable to prove this assumption directly since we were unable to target the same receptors (CD117) with gold NPs and with fluorescent labels, because these were self-excluding processes. However, providing that this is the first experiment with direct correlation of PNBs to the transfection of individual cells, we consider the aim to have been successfully achieved.

All the reported results were obtained for individual cells in order to improve the accuracy of the measurements. The PNB mechanism can also be simultaneously applied to many (10^3 – 10^4) cells with a broad laser beam. Regardless of the beam diameter, the size of PNB will be determined by the NP cluster size, which is a cell-specific feature, and by laser fluence, which is an easily controllable parameter, thus providing the same simultaneous action in many target cells as was described above for individual cells.

4. Discussion

Our results demonstrated for the first time that PNB can be used for selective, effective, and non-toxic gene transfection that can be controlled at the single cell level. Unlike many other photothermal processes, a PNB insulates the environment from laser-induced heat and, as we observed during our previous experiments [51], there is no significant thermal impact associated with PNB generation. Therefore, the mechanism of PNB intracellular targeting should relies on the localized mechanical action of the PNB. The idea of using the mechanical effect of laser pulses for cell transfection is not new and was realized with ultra-short laser pulses through optical breakdown [22]. Vapor bubbles were previously discussed

as a potential means for gene transfection [22,32–41,64]. However, none of these studies detected the bubbles directly, nor correlated the parameters of the bubbles with the transfection effect in individual treated cells. The principal limitation of laser-induced photothermal bubbles relates to their relatively large size that may damage cells [48]. In addition, it is difficult, if not impossible, to selectively generate such macro-bubbles selectively in individual cells. As a result, macro-bubble transfection techniques cannot provide sufficient selectivity and safety, as follows from these previously reported works [22,32–41,64]. In addition, they were unable to provide cell-level guidance of the delivery. In our study, we directly detected the bubbles and correlated them to the process of gene transfection for individual cells. The advantage of using PNBs for gene transfection in general relies on the two main features of PNBs: localized and tunable mechanical action and optical guidance. The optical guidance of PNB action is based on its excellent optical scattering properties that were shown to work *in vitro* and *in vivo* [50,65,66]. We found the correlation between the lifetime of the and the efficacy of gene transfection characterized by the level of GFP-specific fluorescence in the same cells (Fig. 5g and 8). The mechanical action of PNBs can be precisely tuned in individual living with the fluence of the laser pulse, as was shown above (Fig. 4b) and in our previous works [48,50,51]. The single laser pulse procedure excludes prolonged exposure of the cells to physical factors. PNBs can be generated in any cells where gold NPs can be delivered. Our current work included only cells in suspension, but earlier we successfully demonstrated the selective, non-invasive and tunable generation of PNBs in adherent cells [48]. PNB technology can be applied also *in vivo*: the first *in vivo* generation of PNBs (without any gene transfection application) was recently demonstrated by us in several animal models [50,65,66].

A PNB represents an explosion at nanoscale and is therefore potentially dangerous to both cells and tissues. The side effects of PNBs are associated with mechanical and thermal damage to cells and tissues. However, mechanism for PNB generation provides precise control and feedback and thus we are able to generate non-invasive PNBs *in vitro* [46,48] and *in vivo* [50,65]. We have studied in detail the thermal impact of PNBs, and found that the PNB has a unique ability to insulate all heat inside and thus prevents thermal damage to the PNB environment [51,52]. In particular, we have studied the influence of the PNB on the molecular cargo in liposomes and found that the molecules of fluorescent protein were not destroyed during or after PNB generation [49].

5. Conclusions

As a result of our experimental study we found that plasmonic nanobubbles provided a novel means of gene delivery and transfection in specific individual cells by mechanical (non-thermal) injection of DNA plasmid. We demonstrated the following targeting features of plasmonic nanobubbles:

1. A single cell localized targeting mechanism that has a mechanical, non-thermal nature and works through the fast hydrodynamic injection of extracellular molecules into the cellular cytoplasm.
2. A single cell selectivity of targeting was provided by the cell-specific formation of gold NP clusters, and by the localized and controllable nature of plasmonic nanobubbles that were selectively generated in target cells.
3. An ability to treat many cells simultaneously with a single broad laser pulse of a relatively low fluence that induces cell-specific PNBs around gold NP clusters, and does not induce PNBs in non-specific cells even if they uptake non-clustered gold NPs.

4. The plasmonic nanobubbles did not kill the cells because small PNBs were generated and the size of these PNBs was precisely controlled through the laser pulse fluence. In addition, the radiant dose associated with a single laser pulse was relatively small compared to other optical methods.
5. PNBs optically guided the injection process in single cells through the optical scattering of plasmonic nanobubbles.

Plasmonic nanobubbles can therefore be considered an efficient approach to improving gene delivery and transfection, cell therapy and other intracellular molecular delivery methods. The method of plasmonic nanobubbles can be adapted to existing technologies including optical microscopy and flow cytometry, as well as extracorporeal and fiber optic clinical techniques. The successful development of PNB-based gene transfection can lead to safe and effective gene therapy applications in patients with genetic diseases, cancer, and autoimmunity.

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References

1. Kyung Kim T, Eberwine JH. Mammalian cell transfection: the present and the future. *Anal Bioanal Chem.* 2001; 397:3173–3178.
2. Villemejeane J, Mir LM. Physical methods of nucleic acid transfer: general concepts and applications. *Brit J Pharmacol.* 2009; 157:207–219. [PubMed: 19154421]
3. Sommer A, Zhu D, Scharnweber T. Laser modulated transmembrane convection: Implementation in cancer chemotherapy. *J Control Release.* 2010; 148:131–134. [PubMed: 20934473]
4. Kreppel F, Kochanek S. Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. *Mol Ther.* 2008; 16:16–29. [PubMed: 17912234]
5. Pfeifer A, Verma IM. Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet.* 2001; 2:177–211. [PubMed: 11701648]
6. Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med.* 2002; 346:1185–1193. [PubMed: 11961146]
7. Roesler J, Brenner S, Bukovsky AA, Whiting-Theobald N, Dull T, Kelly M, Civin CI, Malech HL. Third-generation, self-inactivating gp91 (phox) lentivector corrects the oxidase defect in NOD/SCID mouse-repopulating peripheral blood-mobilized CD34+ cells from patients with X-linked chronic granulomatous disease. *Blood.* 2002; 100:4381–4390. [PubMed: 12393624]
8. Liu, Dexi; Song, YK. Cationic liposome-mediated transfection *in vivo*. *Gene Ther Mol Biol.* 1998; 2:59–68.
9. Tarahovsky YS. Cell transfection by DNA-lipid complexes – lipoplexes. *Biochemistry (Mosc).* 2009; 74:1293–1304. [PubMed: 19961409]
10. Hunt MA, Currie MJ, Robinson BA, Dachs GU. Optimizing transfection of primary human umbilical vein endothelial cells using commercially available chemical transfection reagents. *J Biomol Tech.* 2010; 21:66–72. [PubMed: 20592869]
11. Schenborn ET, Goiffon V. DEAE-dextran transfection of mammalian cultured cells. *Meth Mol Biol.* 2000; 130:147–153.
12. Zhang Y, Yu L-C. Single-cell microinjection technology in cell biology. *BioEssays.* 2008; 30:606–610. [PubMed: 18478541]

13. Davidson JM, Krieg T, Eming SA. Particle-mediated gene therapy of wounds. *Wound Repair Regen.* 2000; 8:452–459. [PubMed: 11208172]
14. Muangmoonchai R, Wong SC, Smirlis D, Phillips IR, Shephard EA. Transfection of liver *in vivo* by biolistic particle delivery: its use in the investigation of cytochrome P450 gene regulation. *Mol Biotechnol.* 2002; 20:145–151. [PubMed: 11876471]
15. Yoshida S, Kashiwamura SI, Hosoya Y, Luo E, Matsuoka H, Ishii A, Fujimura A, Kobayashi E. Direct immunization of malaria DNA vaccine into the liver by gene gun protects against lethal challenge of *Plasmodium berghei* sporozoite. *Biochem Biophys Res Commun.* 2000; 271:107–115. [PubMed: 10777689]
16. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO J.* 1982; 1:841–845. [PubMed: 6329708]
17. Haas K, Sin W-C, Javaherian A, Li Z, Cline HT. Single-cell electroporation for gene transfer in vivo. *Neuron.* 2001; 29:583–591. [PubMed: 11301019]
18. Schakowski F, Buttgerit P, Mazur M, Märten A, Schöttker B, Gorschlüter M, Schmidt-Wolf IGH. Novel non-viral method for transfection of primary leukemia cells and cell lines. *Genetic Vaccines Ther.* 2004; 2:1–11.
19. Wang M, Orwar O, Weber SG. Single-cell transfection by electroporation using an electrolyte/plasmid-filled capillary. *Anal Chem.* 2009; 81:4060–4067. [PubMed: 19351139]
20. Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet.* 2002; 27:115–134. [PubMed: 12774945]
21. Qiu Y, Luo Y, Zhang Y, Cui W, Zhang D, Wu J, Zhang J, Tu JJ. The correlation between acoustic cavitation and sonoporation involved in ultrasound-mediated DNA transfection with polyethylenimine (PEI) in vitro. *J Control Release.* 2010; 145:40–48. [PubMed: 20398711]
22. Vogel A, Noack J, Huttman G, Paltauf G. Mechanisms of femtosecond laser nanosurgery of cells and tissues. *Appl Phys B.* 2005; 81:1015–1047.
23. Stevenson D, Agate B, Tsampoula X, Fischer P, Brown CTA, Sibbett W, Riches A, Gunn-Moore F, Dholakia K. Femtosecond optical transfection of cells: viability and efficiency. *Opt Express.* 2006; 14:7125–7133. [PubMed: 19529083]
24. Tsukakoshi M, Kurata S, Nomiya Y, Ikawa Y, Kasuya T. A novel method of DNA transfection by laser microbeam cell surgery. *Appl Phys B.* 1984; 35:135–140.
25. Palumbo G, Caruso M, Crescenzi E, Tecce MF, Roberti G, Colasanti A. Targeted gene transfer in eucaryotic cells by dye-assisted laser optoporation. *J Photochem Photobiol B.* 1996; 36:41–46. [PubMed: 8988610]
26. Krasieva TB, Chapman CF, LaMorte VJ, Venugopalan V, Berns MW, Tromberg BJ. Mechanisms of cell permeabilization by laser microirradiation. *Proc SPIE.* 1998; 3260:38–44.
27. Tao W, Wilkinson J, Stanbridge EJ, Berns MW. Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. *Proc Natl Acad Sci USA.* 1987; 84:4180–4184. [PubMed: 3473500]
28. Souhayer JS, Krasieva T, Jacobson SC, Ramsey JM, Tromberg BC, Albritton NL. Characterization of cellular optoporation with distance. *Anal Chem.* 2000; 72:1342–1347. [PubMed: 10740880]
29. Tirlapur U, König K. Targeted transfection by femtosecond laser. *Nature.* 2002; 418:290–291. [PubMed: 12124612]
30. Venugopalan V, Guerra A, Nahen K, Vogel A. Role of laser-induced plasma formation in pulsed cellular microsurgery and micromanipulation. *Phys Rev Lett.* 2002; 88:078103. [PubMed: 11863944]
31. Rhodes K, Clark I, Zatcoff M, Eustaquio T, Hoyte KL, Koller MR, Michael WB, Greulich KO. Cellular laserfection. *Meth Cell Biol.* 2007; 82:309–333.
32. Mohanty SK, Sharma M, Gupta PK. Laser-assisted microinjection into targeted animal cells. *Biotechnol Lett.* 2003; 25:895–899. [PubMed: 12889802]
33. Kohli V, Acker JP, Elezzabi AY. Reversible permeabilization using high-intensity femtosecond laser pulses: applications to biopreservation. *Biotechnol Bioeng.* 2005; 92:889–899. [PubMed: 16189821]

34. Zeira E, Manevitch A, Khatchatourians A, Pappo O, Hyam E, Darash-Yahana M, Tavor EE, Honigman A, Lewis A, Galun E. Femtosecond infrared laser—an efficient and safe in vivo gene delivery system for prolonged expression. *Mol Ther.* 2003; 8:342–350. [PubMed: 12907157]
35. Baumgart J, Bintig W, Ngezahayo A, Willenbrock S, Escobar HM, Ertmer W, Lubatschowski H, Heisterkamp A. Quantified femtosecond laser based opto-perforation of living GFSHR-17 and MTH53 a cells. *Opt Express.* 2008; 16:3021–3031. [PubMed: 18542388]
36. He H, Kong SK, Lee RK, Suen YK, Chan KT. Targeted photoporation and transfection in human HepG2 cells by a fiber femtosecond laser at 1554 nm. *Opt Lett.* 2008; 33:2961–2963. [PubMed: 19079506]
37. Lee S, McAuliffe DJ, Flotte TJ, Kollias N, Doukas AG. Photomechanical transcutaneous delivery of macromolecules. *J Investigative Dermatol.* 1998; 111:925–929.
38. Kodama T, Hambin MR, Doukas AG. Cytoplasmic molecular delivery with shock waves: Importance of impulse. *Biophys J.* 2000; 9:1821–1832. [PubMed: 11023888]
39. Schomaker M, Baumgart J, Ngezahayo A, Bullerdiek J, Nolte I, Escobar HM, Lubatschowski H, Heisterkamp A. Plasmonic perforation of living cells using ultrashort laser pulses and gold nanoparticles. *SPIE Proc.* 2009; 7192:71920U.
40. Yao C, Qu X, Zhang Z, Hüttmann G, Rahmanzadeh R. Influence of laser parameters on nanoparticle-induced membrane permeabilization. *J Biomed Optics.* 2009; 14:054034.
41. Pitsillides CM, Joe EK, Wei X, Anderson RR, Lin CP. Selective cell targeting with light-absorbing microparticles and nanoparticles. *Biophys J.* 2003; 84:4023–4032. [PubMed: 12770906]
42. Umebayashi Y, Miyamoto Y, Wakita M, Kobzyashi A, Nishisaka T. Elevation of plasma membrane permeability on laser irradiation of extracellular latex particles. *J Biochem.* 2003; 134:219–224. [PubMed: 12966070]
43. Kohli V, Elezzabi AY. Laser surgery of zebrafish (*Danio rerio*) embryos using femtosecond laser pulses: optimal parameters for exogenous material delivery and the laser's effect on short- and long-term development. *BMC Biotechnol.* 2008; 8:7–27. [PubMed: 18230185]
44. Brinkmann R, Huettmann G, Roeger J, Roeder J, Birngruber R, Lin CP. Origin of retinal pigment epithelium cell damage by pulsed laser irradiation in the nanosecond to microsecond time regimen. *Laser Surg Med.* 2000; 27:451–464.
45. Braun GB, Pallaoro A, Wu G, Missirlis D, Zasadzinski JA, Tirrell M, Reich NO. Laser-activated gene silencing *via* gold nanoshell-sirna conjugates. *ACS Nano.* 2009; 3:2007–2015. [PubMed: 19527019]
46. Hleb EY, Hu Y, Drezek RA, Hafner JH, Lapotko DO. Photothermal bubbles as optical scattering probes for imaging living cells. *Nanomedicine.* 2008; 3:797–812. [PubMed: 19025454]
47. Lapotko DO. Plasmonic nanoparticle-generated photothermal bubbles and their biomedical applications. *Nanomedicine.* 2009; 7:813–845. [PubMed: 19839816]
48. Hleb EY, Hanna EY, Hafner JH, Lapotko DO. Tunable plasmonic nanobubbles for cell theranostics. *Nanotechnology.* 2010; 21:085102.
49. Anderson LJE, Hansen E, Hleb EY, Hafner JH, Lapotko DO. Optically guided controlled release from liposomes with tunable plasmonic nanobubbles. *J Control Release.* 2010; 144:151–158. [PubMed: 20156498]
50. Wagner DS, Delk NA, Hleb EY, Hafner JH, Farach-Carson MC, Lapotko DO. The in vivo performance of plasmonic nanobubbles as cell theranostic agents in zebrafish hosting prostate cancer xenografts. *Biomaterials.* 2010; 31:7567–7574. [PubMed: 20630586]
51. Lapotko DO. Optical excitation and detection of vapor bubbles around plasmonic nanoparticles. *Opt Express.* 2009; 17:2538–2556. [PubMed: 19219157]
52. Hleb EY, Hu Y, Latterini L, Tarpani L, Lee S, Drezek RA, Hafner JH, Lapotko DO. Plasmonic nanobubbles as transient vapor nanobubbles generated around plasmonic nanoparticles. *ACS Nano.* 2010; 4:2109–2123. [PubMed: 20307085]
53. Lapotko D, Lukianova-Hleb E, Oraevsky A. Clusterization of nanoparticles during their interaction with living cells. *Nanomedicine.* 2007; 2:241–253. [PubMed: 17716124]
54. Lapotko D, Lukianova K, Shnip A. Photothermal responses of individual cells. *J Biomed Optics.* 2005; 10:014006.

55. Hleb E, Lapotko D. Influence of transient environmental photothermal effects on optical scattering by gold nanoparticles. *Nano Letters*. 2009; 9:2160–2166. [PubMed: 19374436]
56. Hleb EY, Hafner JH, Myers JN, Hanna EY, Rostro BC, Zhdanok SA, Lapotko DO. LANTCET: elimination of solid tumor cells with photothermal bubbles generated around clusters of gold nanoparticles. *Nanomedicine*. 2008; 3:647–667. [PubMed: 18817468]
57. Dotti G, Savoldo B, Brenner M. Fifteen years of gene therapy based on chimeric antigen receptors: “are we nearly there yet?”. *Hum Gene Ther*. 2009; 20:1229–1239. [PubMed: 19702437]
58. Aiuti A, Bachoud-Lévi AC, Blesch A, Brenner MK, Cattaneo F, Chiocca EA, Gao G, High KA, Leen AM, Lemoine NR, McNeish IA, Meneguzzi G, Peschanski M, Roncarolo MG, Strayer DS, Tuszynski MH, Waxman DJ, Wilson JM. Progress and prospects: gene therapy clinical trials (part 2). *Gene Ther*. 2007; 14:1555–1563. [PubMed: 17984995]
59. Yam PY, Li S, Wu J, Hu J, Zaia JA, Yee JK. Design of HIV vectors for efficient gene delivery into human hematopoietic cells. *Mol Ther*. 2002; 5:479–484. [PubMed: 11945076]
60. Li M-J, Kim J, Li S, Zaia J, Yee J-K, Anderson J, Akkina R, Rossi JJ. Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Molecular Therapy*. 2005; 12:900–909. [PubMed: 16115802]
61. Feng Y, Wen J, Mike P, Choi DS, Eshoa C, Shi ZZ, Zu Y, Chang CC. Bone marrow stromal cells from myeloma patients support the growth of myeloma stem cells. *Stem Cells Dev*. 2010; 19:1289–1296. [PubMed: 20121456]
62. Petrova H, Min H, Hartland G. Photothermal properties of gold nanoparticles. *Z Phys Chem*. 2007; 221:361–376.
63. Link S, El-Sayed MM. Shape and size dependence of radiative, non-radiative and photothermal properties of gold nanocrystals. *Int Rev Phys Chem*. 2000; 19:409–453.
64. Vogel, A.; Noack, J.; Hüttmann, G.; Paltauf, G. Mechanisms of femtosecond laser nanoprocessing of biological cells and tissues. In: Herman, P.; Hess, W., editors. *Journal of Physics: Conference Series* 59; Proc. Eighth International Conference on Laser Ablation COLA 2005; Institute of Physics Publishing ; 2007. p. 249-254.
65. Hleb EY, Santiago C, Wagner DS, Hafner JH, Lapotko DO. Generation and detection of plasmonic nanobubbles in zebrafish. *Nanotechnology*. 2010; 21:225102. [PubMed: 20453288]
66. Hleb EY, Hafner JH, Myers JN, Hanna EY, Rostro BC, Zhdanok SA, Lapotko DO. LANTCET: elimination of solid tumor cells with photothermal bubbles generated around clusters of gold nanoparticles. *Nanomedicine*. 2008; 3:3, 647–667.

Figure 1.

Principle of intracellular molecular targeting (IMT) with plasmonic nanobubble (PNB): (a) selective formation of gold NP cluster in target cell; (b) generation of PNB with a short single laser pulse (green arrows) and mechanical disruption (perforation) of the cellular membrane by the PNB; optical scattering of the probe laser radiation (red arrows) guides the PNB action; (c) hydrodynamic injection of the extracellular molecules (blue) into cellular cytoplasm by the micro-jet that emerges during the collapse of the PNB.

Figure 2.

A,e – Confocal bright field images of cells (top – J32, bottom – JRT3-T3.5); b, f - confocal scattering images of gold NPs (shown in red) overlaid on the differential interference contrast images of the cells, bright red dots show the clusters of NPs; c, g – merged bright field and scattering images, and d, h – profiles of scattering image amplitude of NP clusters as shown in b and f, respectively. Scale bar is 7 μm .

Figure 3.

A,c: Time-resolved scattering optical images of individual cells show large PNBs only in target J32 cell (a, the pixel image amplitude shown in the gray scale), during its exposure to the single pump laser pulse at 532 nm, 0.5 ns, while the test cell JRT3-T3.5 yielded a very small PNB under the same NP and laser treatment (c); b, d: corresponding time responses obtained simultaneously for the same cells show the PNB size through the duration of these signals. Dashed lines show cell borders. Scale bar is 7 μ m.

Figure 4.

PNB parameters in the populations of cultured cells (■ – J32, □ – JRT3-T3.5) after treatment with gold conjugates and exposure to a single laser pulse: PNB generation probability (a) and (b) PNB lifetime.

Figure 5.

A: Bright field (a, c, e) and GFP fluorescence (b, d, f) image of individual cells: a, b - J32; c, d - JRT3-T3.5 (48 hours after NP and PNB treatment); e, f - positive control. Scale bar is 7 μm . G: The diagram shows PNB size (lifetime, axis x) and the amplitude of GFP fluorescence (axis Y) obtained for individual cells and the population-averaged for OKT3 positive cells J32 (red) and for OKT-negative cells JRT3-T3.5 (black): ● - 0 hours, triangle - 24 hours, ○ - 48 hours. Black horizontal lines show the levels of the fluorescent amplitude for the GFP-positive and negative controls.

Figure 6. Bright field (a, c) and GFP fluorescence (b, d) images of the individual CD34-positive cells in the test sample with CD117 cells 48 hours after treatment with NP-mAb conjugates and PNB: a, b - the cell with high fluorescence (subpopulation 1); c, d - the cell with low fluorescence (subpopulation 2). Scale bar is 7 μ m.

Figure 7.

A: The levels of the image amplitude of GFP-specific fluorescence as measured immediately after the PNB treatment (0 hours), and 24 hours and 48 hours after the PNB treatment for CD34-positive cells without CD117-positive cells (control, black) and for the two sub-populations of CD34-positive cells in the test sample with CD117 cells (grey and white). B: The levels of CD117-positive cells (white), PNB generation probabilities (grey) and GFP-positive cells (black) in the test and control samples.

Figure 8.

The diagram shows PNB size (lifetime, axis X) and the amplitude of GFP fluorescence (axis Y) obtained for individual CD34-positive cells and the population-averaged for CD117-positive cells (test, red) and for CD117-negative cells (control, black): ● – 0 hours, triangle – 24 hours, ○ – 48 hours.

Table 1

Transfection efficacy (%) and viability (%) of the PNB treated cultured cells

Time after PNB treatment	0 hours		24 hours		48 hours	
	Transfection efficacy	Cell viability	Transfection efficacy	Cell viability	Transfection efficacy	Cell viability
I32	0	99	0	89	74	84
IRT3-T3.5	0	99	0	91	0	91

Table 2

PNB parameters in individual human stem cells

	CD34-positive cells with a sub- population of CD117-positive cells (test)	CD34- positive cells without CD117-positive cells (control)
PNB generation probability, %	13.0	0
PNB lifetime, ns	57±33	0

Table 3
Transfection efficacy (%) and viability (%) of the PNB treated human stem cells

Time after PNB treatment Parameter	0 hours		24 hours		48 hours	
	Transfection efficacy	Cell viability	Transfection efficacy	Cell viability	Transfection efficacy	Cell viability
CD34-positive, CD117-positive	0	99	43	90	71	83
CD34-positive, CD 117-negative	0	99	0	91	0	91