Spatially, Temporally, and Quantitatively Controlled Delivery of Broad Range of Molecules into Selected Cells through Plasmonic Nanotubes

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We present an in vitro platform for delivering a broad range of molecules into the intracellular compartment with single cell selective control and high parallelization potential. The delivery is achieved by gold nanotubes that are tight adhering to cells on one side and directly connected to a microfluidic channel on the other side. The physical mechanism relies on the generation of nanoshockwaves opening transient nanopores into the cell membrane in close proximity to the nanotube end. The 3D geometry of the gold nanotubes, which protrude from the substrate plane, promotes a very good seal between the nanotubes and the cell membrane. It results in a very good reproducibility and reliability of the poration mechanism (success rate ≥95%). Moreover, the method does not let any molecule in the culture bath enter the cell, unlike other poration techniques. Finally, the membrane reforms spontaneously in a few minutes thus restoring the initial condition with no side effects. The method is easy-to-use, does not require specific skills, and can be directly integrated into the majority of biological protocols for in vitro investigations, electrophysiological measurements, and plasmon-enhanced spectroscopies.

The delivery of molecules across the cell membrane and into the intracellular compartment is a fundamental requirement of molecular biology, currently accomplished by many different in vitro methods.[1–5] Broadly speaking, current methods can be divided into two main categories: "chemical methods," in which biomolecules recognized by the membrane are used in a Trojan-horse strategy that promotes a spontaneous uptake into the cell; and "physical methods," in which micropipettes or electrodes are used to transiently breach the membrane (microinjection and electroporation). Despite the general effectiveness of these methods, there are numerous limitations. For instance, chemical methods allow a large number of cells to be targeted simultaneously, but often require the delivered molecule to be chemically conjugated to its carrier, necessitating specific protocols for each molecule used. Viral vectors provide high transfection efficacy for delivering nucleic acids, but are limited in terms of the length of the inserted nucleic acids, and can also present biosafety concerns or cell tropism.

On the other hand, physical methods like electroporation[6] and microinjection[7] allow a broad range of molecule to be introduced into the cell without the need of chemical conjugation. However, microinjection is difficult to automate, and electroporation presents some concerns related to potential damage. Some difficulties can be partially overcome by culturing cells in adhesion with arrays of alumina nanostraws[8] or vertical silicon nanowires[9,10] which represent advanced versions of electroporation and microinjection, respectively.

However, they offer a limited control on shape and size of nanostructures and they are randomly arranged on the substrate, thus making the adhesion of the nanostraw (or vertical nanowire) to the cell membrane variable from a structure to another, even in the same cell. The latter can induce high variability in the poration process (both on pore size and membrane reforming time), especially when single cell experiments are performed. Furthermore, they generally do not allow targeting of specific cells or subsets of cells. For example, to access selected single cells or cell groups for electroporation in an in vitro culture, one would need a dense and complex array of electrical leads.

Single cell technologies can provide new and exciting experimental opportunities for unveiling cell individuality that is hidden in experiments carried out on a large ensemble. A step behind this direction is represented by ultrafast optoporation that is an alternative to the classical methods of intracellular delivery.[11,12] Very recently, optoporation was also achieved in the microsecond regime by generating microbubbles oscillating in size.[13] In this case, the induced shear stress creates transient pores in the cell membrane thus enabling delivery of molecules. Some groups also investigated the capability of enhancing optical poration with plasmonic nanoparticles.[14] The introduction of plasmonic systems in this kind of experiments can be of great interest since it enables the exploitation of Raman spectroscopy that provides a deeper insight into the cellular biochemical environment and its local changing at the molecular level.[15–19]

Finally, both chemical and physical methods do not afford precise control over the amount of molecules effectively delivered inside the cell. Moreover, they allow virtually any molecule present in the extracellular fluid to pass into the cell thus inducing nonspecific uptake.

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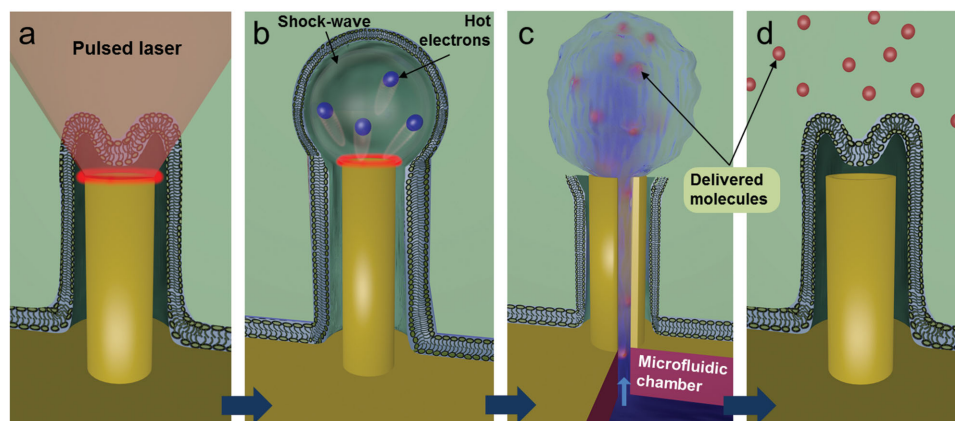E-mail: Francesco.deangelis@iit.it

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Figure 1. Sketches representing the delivery method: a gold plasmonic nanotube is fabricated on an underneath microfluidic channel, then cells are cultured on the nanotubes. a) A laser pulse is exploited to excite the nanotube. b) The generated pressure waves are able to locally open a transient nanopore. c) Molecules are delivered to the intracellular compartment through the nanopore. d) The nanopore spontaneously closes in few minutes. In that time window, the microfluidic channel is used to deliver the desired molecules.

In summary, an ideal platform for intracellular delivery should be easily scalable from single cells to large ensemble experiments (scalability). It should enable the delivery of any molecule (wide use) in a well-defined amount (control on quantity) to the selected cell or group of cells (control on space) in an arbitrary time-window (control on time). Furthermore, it should be easy to use with negligible side effects.

Here, we present an innovative technique that allows any molecule to be delivered into a selected cell or group of cells within a population of in vitro cultured cells, which is capable of overcoming most of the limitations introduced above.

For the sake of clarity, we first summarize the overall concept and results, and then we describe in more detail the experiments we performed to prove the validity of the method. As sketched in **Figure 1**, hollow plasmonic nanotubes[20] are combined with a microfluidic channel embedded into the substrate. Cells are cultured on the top of the device, which appears as a standard gold substrate with nanotubes protruding from the substrate plane. As described thereafter, a short pulse laser is focused on a nanotube to excite a strong and confined electric field at the nanotube ends by means of plasmonic enhancement. Such a highly enhanced electric field causes the expulsion of electrons from the gold nanotube tip to the surrounding water. Electrons are then accelerated by the laser field to generate nanoshockwaves that locally opens transient nanopores on the cell membrane, without generating microbubbles potentially dangerous for cells. The underlying microfluidic chip is then used to administer the desired molecule to the selected cell. No pressure difference is needed for delivering molecules (diffusion-mediated process). The process ends with spontaneous membrane reforming that occurs in a few minutes and without side effects.

The details of the technique are as follows. A plasmonic nanotube made of gold is fabricated on a silicon nitride membrane (1.1 μm in height, 180 nm in diameter, and 90 nm inner nanochannel; see also **Figure 2** and Section S1 in the Supporting Information).

The nanotube is excited with 8 ps laser pulses with a peak power of 5×10^8 W cm^{-2} at $\lambda = 1064$ nm, through a microscope objective (NA = 0.8). Single pulses have energy of 62.5 pJ, and were repeated at 80 MHz for 10 ms (with an average power of 5 mW). The laser pulse will excite surface plasmon polaritons which are able to decay into highly energetic electrons, called "hot electrons." Hot electrons are recently attracting a lot of attention for their unique features.[21] Among them, it is well known that they are very effective in being injected into a semiconductor material.[22–24] In laser nanosurgery, water is usually treated as an amorphous semiconductor[25,26] in which free-electron density can be generated by exciting electrons from the valence to the conduction band. This process requires activation energy equal to 6.5 eV, whereas the energy necessary to remove an electron from gold in the presence of water is 3.7–2.2 eV,[27–29] thus making the plasmonic process strongly favored and efficient. Once a free electron is promoted to the conduction band of water, it can gain kinetic energy (acceleration) by absorbing photons in a process called "inverse bremsstrahlung." As a result of that process, water molecules are accelerated to generate a shockwave that opens transient nanopores into the cell membrane (for details see Section S2 in the Supporting Information and specialized reviews).

Following this approach, we cultured NIH/3T3 cells on 3×3 arrays of gold nanotubes (see **Figure 3**a,b), and placed the system on an inverted microscope. The poration procedure was performed by irradiating a single antenna with a train of focused laser pulses (Full Width at Half Maximum (FWHM) ≈ 1 μm) at an average power of 5 mW. Immediately after the laser poration, we injected propidium iodide (PrId, 1.5×10^{-3} concentration) from the microfluidic channel underneath the nanotube. Propidium iodide is a membrane impermeable dye that binds to double stranded DNA: the appearance of fluorescence signal coming from the cell indicates the introduction of the dye into the intracellular environment. Optical images from a typical experiment are shown in Figure 3c,f, in which three cells were sequentially porated.

The three cells were sequentially porated and injected over a period of a few minutes, through manual operation (point and click). However, the approach can be automated by proper software that, by analyzing an optical image, recognize the
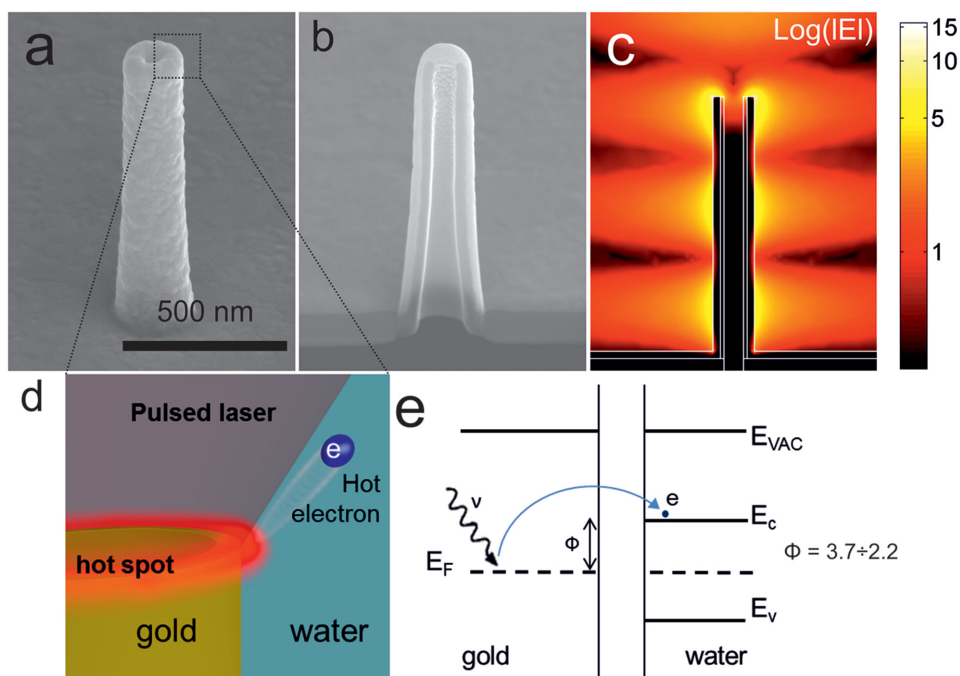
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Figure 2. SEM (Scanning Electron Microscopy) images of a) gold nanotube (1.1 µm in height, 180 nm in diameter, and 90 nm inner nanochannel) fabricated on a silicon nitride membrane entirely covered with a 30 nm thick film of gold. b) Cross section showing the underneath microfluidic channel. c) FEM (Finite Elements Method) simulations of the electric field enhancement due to the laser excitation of plasmons. d) Sketch representing "hot electron" emission from gold to water (cell membrane not represented for simplicity). e) Associated band diagram.
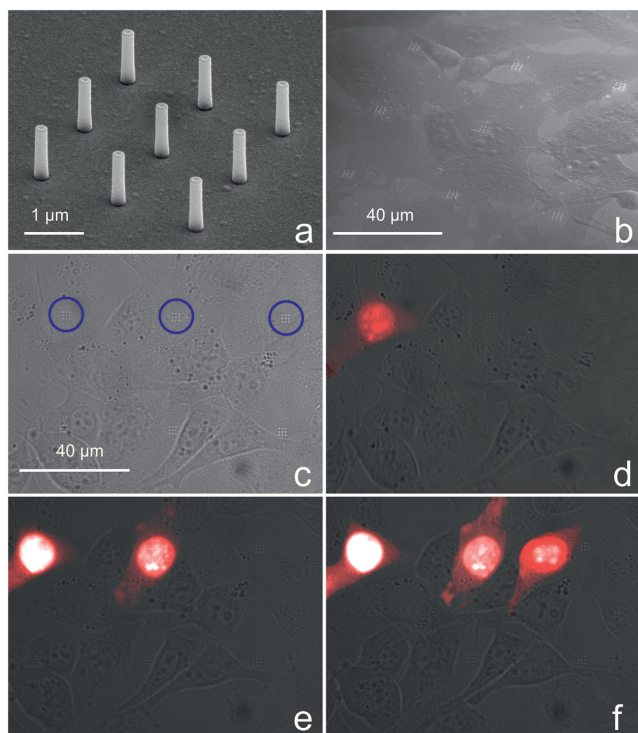

Figure 3. SEM images of a) 3 × 3 array of nanopillars and b) NIH/3T3 cells cultured on arrays. c) Optical microscopy and d–f) fluorescence images of the same field of view showing selective poration of contiguous cells.

nanotubes, shots on them, and then move to the next field of view. In such a procedure, the minimum time interval between two poration events depends on the scanning speed of the optical microscope. Since time interval can be as fast as a few milliseconds, it would be possible to porate 10^5 cells h^{-1} or even more.

The type and the amount of molecules delivered into the cells can be controlled by tuning the injection time (time when molecules are fluxing in the microfluidic channel) and the composition of solution flowing into the microfluidic channel. We point out that no pressure drop is applied to the microfluidic channel, as in an ion channel molecules spontaneously diffuse into the cell as due to the concentration gradient.

To examine whether a transient nanopore is formed, we carried out experiments delaying the injection of the dye inside the membrane relative to the poration time. In healthy cells, both optoporation[11] and electroporation[6] processes lead to the generation of transient holes that tends to reduce their dimensions and close ≈10 min after the poration event, presumably by the rearrangement of the lipidic bilayer.[30] When PrId was introduced into the microfluidic channel immediately after laser irradiation, or within 5 min, the selected cells accumulated the fluorescent dye (effective delivery, **Figure 4**).

However, when the injection was done 10 min after the poration, we found a reduction in injection efficiency, thus suggesting a partial membrane reforming (pore closing). After 15 min, a few cells evidenced a modest coloration. This suggests that the opening in the membrane was transient, with a lifetime of ≈10 min and small variations from one cell to
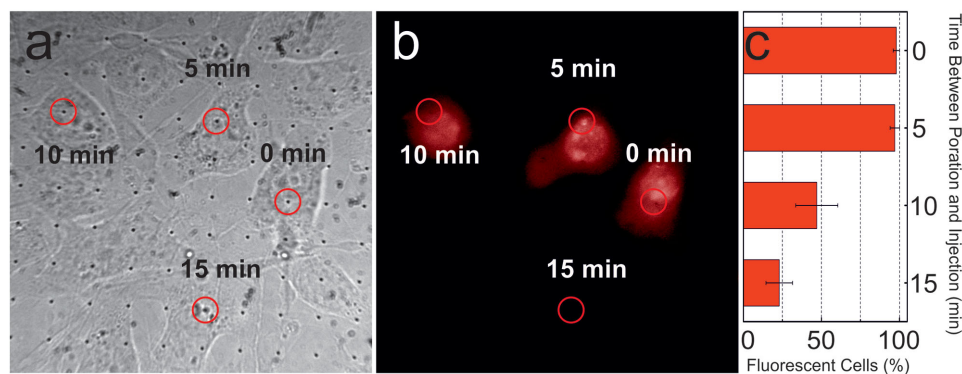
Figure 4. a) Optical image showing cell porated at different times before injection of propidium iodide and b) corresponding fluorescence image. c) Histogram reporting the percentage of fluorescent cells as a function of time between poration and injection on a statistics of 12 samples.

another. Also, these results provide an important evidence of nonspecific damage of the laser pulse to the cells, or leakage of PrId into cells. For example, if the plasmonic process or the laser pulse itself nonspecifically damaged or even killed the cells, they would tend to take up PrId 15 min after the laser illumination.

Another important question to address is if a "seal" occurs between the nanotube and the cell membrane after the nanopore opens, and if it is tight enough to exclude the leakage of molecules from the extracellular medium. To verify that, we administrated PrId directly to the cell culture medium ("not" in the microfluidic channel), and then opened transient nanopores on the nanotube tip by laser pulses. No intracellular delivery of PrId was observed in this case, thus confirming an exclusive seal between the cell membrane and the nanotube body (see also Section S5 in the Supporting Information). The observation that the technique does not let any molecule in the bath enter the cell, unlike other poration techniques, is another confirmation of the excellent control allowed by the technique. In consequence, the proposed method enables radically new experiments currently impossible with existing physical methods.

The main advantage of the exclusive seal is that it enables to quantify and tune the amount of delivered molecules. Since the intracellular access time varied from cell to cell (on the order of 10–15 min) we can set the injection time to a shorter interval to equalize the amount delivered among cells. For instance, by using the microfluidic chip a period of 5 min can be set. On the basis of the geometrical parameters of the nanotube channel, we theoretically evaluated the delivered amount (see Section S4 in the Supporting Information): for a dye concentration of 1.5×10^{-3} M, and an injection time of 5 min, we have estimated an amount of about 3×10^8 molecules of PrId delivered. For a cell of average size, it corresponds to a concentration of about 3×10^{-5} M, near to the concentrations typically used for staining assays.

To examine the overall biocompatibility of our method, including the effect of both nanostructures and laser illumination, we performed additional tests. By using Calcein AM green dye to indicate the vitality of the cells, we found that cells retained their viability even 2 days after the illumination/poration treatment, and continued to show normal adhesion and morphology (see viability essay, see Section S6 in the Supporting Information).

The advantages of using plasmonic nanostructures with respect to conventional optoporation appear evident by looking at the numbers involved. The average power used in our experiments is an order of magnitude lower than that usually employed in optoporation, thus reducing thermal effects and potential damage. Moreover, our nanotubes protrude from a substrate covered with a gold layer that conducts heat much better than water. Hence, the generated heat is expected to be rapidly dissipated into the substrate rather than into the cell thus further reducing potential heating of the cells. Concerning the plasmonic enhancement, we have already demonstrated that, thanks to the particular 3D geometry, the fabricated out-of-plane nanostructures provide very good performances.[20,31] For the geometries reported above, we calculated an enhancement factor of 15 at $\lambda = 1064$ (see Section S2 in the Supporting Information).

The particular geometry of the exploited nanotubes offers different important advantages also with respect to other physical or chemical methods. As we have shown, cells spontaneously grab the nanotubes thus inducing a tight adhesion between the membrane and the whole nanotube body. Such a tight adhesion provides a well-defined and predictable contact point between the membrane and the plasmonic nanoantennas. In contrast, the adhesion points of nanoparticles to the cell membrane are random, unpredictable, and not easily observable with a common optical microscope.

We found that such a very good sealing with the nanotube side wall is preserved even when the membrane is porated on the top of the nanotube (as sketched in Figure 1). It means that when the pore is open, only the molecules present in the microfluidic channel can access the intracellular environment, whereas the molecules present in the bath cannot enter the cell. This fact has some immediate important consequences: (i) it enables to equalize the amount of molecules delivered to different cells; (ii) it prevents nonspecific uptake from the bath; (iii) it enables the capability of quantifying and tuning the amount delivered; (iv) it enables a very good reproducibility and reliability of the poration mechanism (efficacy or success rate ≥95%). Potential delivery agents include a broad range of nanoagents such as genetic material, nanoparticles, or arbitrary molecules, independently of their size, charge, folding, or molecular structures. Even small ions (K^+, Na^+, and Cl^-) could be in principle delivered without changing the extracellular

concentration. When desired, also complex solutions containing a mixture of molecules can be delivered in consecutive steps without affecting the extracellular bath. These are the unique features not supported by other poration methods.

In summary, we presented a straightforward method for delivering arbitrary molecules into cells by using microfluidic chips refined with plasmonic nanotubes, and standard confocal microscope equipped with short laser pulses in the IR range. In principle, the approach can be used on any cell line that adheres to the substrate. Potential delivery agents including genetic material, nanoparticles, or complex solutions could be injected with no need of dedicated protocols as in chemical methods or viral transfections. At the current state, the technique can access single cells within a population; however, by proper optimization it could be automatized to process 10^5 cells h^{-1}.

The very good seal between the nanotubes and the cell membrane ensures very specific delivery of molecules, thus preventing nonspecific uptake from the extracellular fluids as in the majority of delivery methods. Importantly, the tight seal enables to quantify and tune the amount of delivered molecules.

We also notice that other very promising developments are conceivable. For instance, these plasmonic nanotubes are also very effective for spectroscopic investigations such as Raman scattering[20,32] that give a deep insight into the biochemical processes occurring on the cell membrane or on the intracellular environment.[18] Furthermore, by using standard lithographic techniques, arrays of planar electrodes can be achieved in combination with the nanotubes. In this way, the gold nanotubes could be used to carry out electrophysiological measurements in combination with the delivery.[33] Finally, we notice that the same approach can be diverted to collect intracellular fluids from single selected cells and analyze them with ultrasensitive methods such as mass spectroscopy or quantitative PCR (Polymerase Chain Reaction) thus enabling real-time monitoring of gene expression and protein content with scalable resolution up to single cell analysis.

Supporting Information

Acknowledgements

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