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A vector-free microfluidic platform for intracellular delivery

Armon Sharei, Janet Zoldan, Andrea Adamo, Woo Young Sim, Nahyun Cho, Emily Jackson, Shirley Mao, Sabine Schneider, Min-Joon Han, Abigail Lytton-Jean, Pamela A. Basto, Siddharth Jhungjunwala, Jungmin Lee, Daniel A. Heller, Jeon Woong Kang, George C. Hartoularos, Kwang-Soo Kim, Daniel G. Anderson, Robert Langer, and Klavs F. Jensen

Intracellular delivery of macromolecules is a challenge in research and therapeutic applications. Existing vector-based and physical methods have limitations, including their reliance on exogenous materials or electrical fields, which can lead to toxicity or off-target effects. We describe a microfluidic approach to delivery in which cells are mechanically deformed as they pass through a constriction 30–80% smaller than the cell diameter. The resulting controlled application of compression and shear forces results in the formation of transient holes that enable the diffusion of material from the surrounding buffer into the cytosol. The method has demonstrated the ability to deliver a range of material, such as carbon nanotubes, proteins, and siRNA, to 11 cell types, including embryonic stem cells and immune cells. When used for the delivery of transcription factors, the microfluidic devices produced a 10-fold improvement in colony formation relative to electroporation and cell-penetrating peptides. Indeed, its ability to deliver structurally diverse materials and its applicability to difficult-to-transfect primary cells indicates that this method could potentially enable many research and clinical applications.

Intracellular delivery of macromolecules is a critical step in therapeutic and research applications. Nanoparticle-mediated delivery of DNA and RNA, for example, is being explored for gene therapy (1, 2), while protein delivery is a promising means of affecting cellular function in both clinical (3) and laboratory (4) settings. Other materials, such as small molecules, quantum dots, or gold nanoparticles, are of interest for cancer therapies (5, 6), intracellular labeling (7, 8), and single-molecule tracking (9).

The cell membrane is largely impermeable to macromolecules. Many existing techniques use polymeric nanoparticles (10, 11), liposomes (12), or chemical modifications of the target molecule (13), such as cell-penetrating peptides (CPPs) (14, 15), to facilitate membrane poration or endocytotic delivery. In these methods, the delivery vehicle’s efficacy is often dependent on the structure of the target molecule and the cell type. These methods are thus efficient in the delivery of structurally uniform materials, such as nucleic acids, but often ill-suited for the delivery of more structurally diverse materials, such as proteins (16, 17) and some nanomaterials (7). Moreover, the endosome escape mechanism that most of these methods rely on is often inefficient; hence, much material remains trapped in endosomal and lysosomal vesicles (18). More effective gene delivery methods, such as viral vectors (19, 20), however, often risk chromosomal integration and are limited to DNA and RNA delivery.

Membrane poration methods, such as electroporation (21, 22) and sonoporation (23), are an attractive alternative in some applications. Indeed, electroporation has demonstrated its efficacy in a number of DNA (24) and RNA (25) delivery applications for previously difficult-to-transfect primary cells. However, this method can cause cell death and has been shown to damage sensitive materials such as quantum dots, which aggregate due to exposure to electric fields (8). There have also been limited reports of successful protein delivery by this mechanism (26, 27). Microinjection, is perhaps the most direct method of delivering material to the cell cytoplasm and is not limited to DNA or RNA, but often ill-suited for the delivery of more structurally diverse materials such as proteins (16, 17) and some nanomaterials (7). Moreover, the endosome escape mechanism that most of these methods rely on is often inefficient; hence, much material remains trapped in endosomal and lysosomal vesicles (18). More effective gene delivery methods, such as viral vectors (19, 20), however, often risk chromosomal integration and are limited to DNA and RNA delivery.

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The authors declare no conflict of interest.

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The delivery performance depends on cell speed and constriction design. Constriction dimensions (Fig. S1) are denoted by numbers (e.g., 10 μm – 6 μm x 5) such that the first number corresponds to constriction length, the second to constriction width and the third (if present) to the number of constrictions in series per channel. (A) Delivery efficiency and (B) cell viability 18 h after treatment as a function of cell speed for 40 μm – 6 μm (C), 20 μm – 6 μm (C), and 10 μm – 6 μm x 5 (A) device designs. Efficiencies and viabilities were measured by flow cytometry after propidium iodide staining. For information regarding cell recovery rates, refer to SI Note S1. All data points were run in triplicate, and error bars represent 2 SDs.
All data points were run in triplicate, and error bars represent 2 SDs. Applicability across cell types. To investigate the versatility of the technique, we assessed its ability to deliver model dextran molecules to several cell types that are traditionally difficult to transfect, especially immune cells and stem cells. Fluorescently labeled 70- and 3-kDa dextran were chosen for these experiments because they are similar in size to many protein and siRNA molecules respectively, easy to detect by flow cytometry, and have minimal surface binding effects as they are negatively charged. Using various device designs, we were able to deliver dextran molecules to newborn human foreskin fibroblasts (NuFFs) (Fig. 4A), primary murine dendritic cells (Fig. 4B), and embryonic stem cells (Fig. 4C). These experiments yielded minimal loss (<25%) in cell viability (Fig. 4 A–C), and preliminary results in murine embryonic stem cells indicate that the method does not induce differentiation (Fig. S4). In further studies, we isolated white blood cells (buffy coat layer) from murine blood by centrifugation and treated them with the device. B cells, T cells, and macrophages, as differentiated by antibody staining, indicated successful delivery of both 3- and 70-kDa dextran (Fig. 4 D and E, and Figs. S5–S7).

The preliminary evidence for a poration- and diffusion-based mechanism of delivery (Fig. 3 A–C) and the functionality of delivered materials (Fig. 3D) would indicate that these dextran

Fig. 3. Diffusive delivery mechanism. (A) Scans of different horizontal planes of a HeLa cell after the delivery of cascade blue-conjugated 3-kDa dextran, as measured by confocal microscopy. Note that 3-kDa dextran is small enough to enter the nuclear envelope (43). Scans read from top to bottom, and then left to right, where the top left is at z = 6.98 μm and bottom right is at z = 6.7 μm. (Scale bar: 6 μm.) (B) Live-cell delivery efficiency of 10 μM − 6 μM (□), 20 μM − 6 μM (○), 30 μM − 6 μM (△), and 40 μM − 6 μM (◇) devices. The time axis indicates the amount of time elapsed from initial treatment of cells before they were exposed to the target delivery solution. All results were measured by flow cytometry 18 h after treatment. (C) Average intensity of the delivered cell population normalized by untreated cells to control for autofluorescence. Fluorescein-conjugated 70-kDa dextran and cascade blue-conjugated 3-kDa dextran are delivered to the cell (cycles 1 and 3) and removed from the cell (cycle 2) in consecutive treatment cycles. The control represents cells that were only exposed to the delivery solution and not treated by the device. (D) Gene knockdown, as a function of device type and cell speed, in live destabilized GFP-expressing HeLa cells 18 h after the delivery of anti-eGFP siRNA at a delivery concentration of 5 μM. Lipofectamine 2000 was used as a positive control and scrambled controls were run at 500 μM/s on a 10 μM device to deliver 3- and 70-kDa dextran. (Fig. S2 A and B) would indicate that these dextran

with PBSs and treated again in the absence of dextran (cycle 2), and finally treated a third time in the presence of 3- and 70-kDa dextran (cycle 3). The changes in normalized fluorescence intensity demonstrate a net diffusion of dextran into the cells during the first cycle, out of the cells during the second, and back in during the third (Fig. 3C). These results are thus consistent with the diffusive delivery hypothesis. A simplified, 2D diffusion model was developed in COMSOL to simulate the passive diffusion of material into a cell across a porous membrane (Fig. S3 and SI Note S1). Using literature values for particle diffusivities inside and outside the cell cytoplasm (36), we were able to qualitatively re-create the experimental results of Fig. 3C with diffusion as the only mode of mass transfer. Moreover, by fitting our experimental data to this model, we estimate that the final concentration of delivery material in the cell cytosol is within 10–40% of the buffer concentration. Furthermore, we demonstrated the functionality of the delivered materials by producing dosage-dependent, sequence-specific fluorescence knockdown in GFP-expressing HeLa cells (Fig. 3D and Fig. S2C). Although Lipofectamine 2000, a commercially available transfection reagent, achieved greater knockdown efficiencies, one must account for the prolonged delivery period of Lipofectamine particles (cells were incubated overnight) relative to the device’s 2- to 5-min poration window. Device design and operating parameters were not optimized for siRNA delivery before performing these experiments.
delivery data should be representative of the expected cytosolic delivery efficiency for a protein or siRNA molecule of interest that is of similar size. Device designs have not been optimized for any of the aforementioned cell types, and we thus expect that further studies will yield improvements in viability and delivery efficiency. For a list of cell types that have successfully been treated using this technique and guidelines to designing new devices for specific cell types please refer to SI Text (Tables S1 and S2, and SI Note S2).

Enabling Delivery Platform. To illustrate our method’s potential in addressing current delivery challenges, we conducted a number of proof-of-concept experiments in possible applications ranging from cell reprogramming (4) to carbon nanotube-based sensing (37). In addition to the application-specific materials detailed below, this method has demonstrated the successful delivery of Apolipoprotein E, BSA, and GFP-plasmids. The delivery of PEG1000-coated, 15-nm gold nanoparticles was verified by tunneling electron microscopy (TEM) of HeLa cells (Fig. 5 A and B). The nanoparticles appear to be mostly unaggregated and are not visibly sequestered into endosomes. In these images, we also observed tentative evidence for various defects in the cell cytoplasm, which could be the proposed holes responsible for delivery. We have also demonstrated high-throughput, noncytotoxic delivery of quantum dots directly to the cell cytosol (38)—a goal that current techniques have struggled to achieve. Furthermore, we were able to verify the successful delivery of carbon nanotubes (37) (encapsulated by a DNA oligonucleotide) by flow cytometry (Fig. 5C) and Raman spectroscopy (Fig. 5D). Antibodies to tubulin were also delivered (Fig. 5 E and F) using this technique, yielding a diffuse distribution throughout the cell that would be consistent with cytosolic delivery. The aforementioned materials are currently difficult to deliver to the cell cytosol and each material often requires a specialized modification to facilitate delivery. In our work, all four materials were delivered to HeLa cells using the same set of conditions on a 10 μm – 6 μm × 5 device.

Efficient delivery of proteins to primary cells could enable several therapeutic applications. A challenge in cell reprogramming (4), for example, is the inefficiency of current CPP-based protein delivery methods. We examined our ability to deliver four transcription factors (Oct4, Sox2, c-Myc, and Klf4) to human fibroblast cells and compared our results to a CPP method (4) (Fig. 6A). Our results show that, in addition to not relying on endocytosis, which can leave much material trapped in endosomes, delivery by rapid mechanical deformation yields significantly higher delivery efficiency for all four proteins. Confocal imaging of cells treated by the device indicated that the transcription factors appear to successfully localize to the nucleus (Fig. 6B). Finally, to investigate the system’s ability to affect gene transcription rates through the delivery of these proteins, we partially replicated a previous reprogramming study using commercially available (Stemgent) recombinant proteins (39). Briefly, NuFF cells were treated in the presence of recombinant c-Myc, Klf4, Oct4, and Sox2 for four cycles, spaced 2 d apart. The emergence of transformed colonies was monitored over a 1-mo period after the last treatment (Fig. 6C). The device was able to generate an average of 150 transformed colonies per plate relative to 11 and 2 colonies for electroporation and CPPs, respectively (Fig. S8). These colonies expressed embryonic stem cell markers, such as Oct4, SSEA-4, Tra-1-60, and Tra-1-81, and were capable of differentiating into all three germ layer cell types (Fig. 6 D–G and Fig. S8). These results suggest that transcription factors delivered by the microfluidic device are capable of affecting gene/protein expression more effectively than existing alternatives such as CPPs and electroporation.

Discussion

In the proposed intracellular delivery method, we hypothesize that transient holes are formed by rapid mechanical deformation of a cell as it passes through a microfluidic constriction. Our data support this notion by demonstrating diffuse cytosolic staining (Fig. 3A), siRNA functionality (Fig. 3D), and the bidirectional movement of material across the disrupted membrane (Fig. 3C).

![Fig. 5. Nanomaterial and antibody delivery. (A and B) TEM images of gold nanoparticles (some indicated by arrows) in cells fixed ~1 s after treatment by a 10 μm – 6 μm × 5 device (SI Note S3). (Scale bars: 500 nm.) (C) Delivery efficiency and viability of HeLa cells treated with a 10 μm – 6 μm × 5 device to deliver Cascade Blue-labeled 3-kDa dextran and Cy5-labeled, DNA-wrapped, carbon nanotubes. (D) Bright-field cell images overlaid with Raman scattering in the G-band (red) to indicate delivery of carbon nanotubes in treated cells (Left) vs. endocytosis (Right). (Scale bars: 2 μm.) (E) Fluorescent micrograph of a HeLa cell 18 h after delivery of Cascade Blue-labeled 3-kDa dextran (Center) and antibodies to tubulin with an Alexa Fluor 488 tag (Right). (Scale bars: 3 μm.) (F) Delivery efficiency and viability of HeLa cells treated with a 10 μm – 6 μm × 5 device, at 500 mm/s, to deliver Alexa Fluor 488-labeled anti-tubulin antibodies. Delivery efficiency at different antibody concentrations is compared with an endocytosis control at 100 μg/mL and untreated cells.](image-url)
and we have illustrated the system to electroporation and CPP delivery of transcription factors further demonstrates relatively high viability in most applications and there is no underlying mechanism by which sensitive payloads, such as some of which are challenging to use with current methods, to a variety of difficult-to-transfect cell types, including stem cells and immune cells. By providing flexibility in application and obviating the need for exogenous materials or electrical fields, this method could potentially enable new avenues of disease research and treatment. Indeed, our work has demonstrated this system’s ability to deliver carbon nanotubes, quantum dots (38), and antibodies to live cells—applications that could enable new sensing and imaging modalities—and we have illustrated the system’s superior performance, relative to current methods, in applications such as transcription factor delivery for reprogramming.

**Materials and Methods**

**Device Fabrication and Mounting System.** The silicon-based devices are fabricated at the Massachusetts Institute of Technology microfabrication facility using photolithography and deep reactive ion etching techniques. In this process, 6" silicon wafers with a 450-μm thickness are treated with hexamethyldisilazane, spin coated with photoresist (OOG934; Fujifilm) for 60 s at 3,000 rpm, exposed to UV light (EV1; EVG) through a chrome mask with the constriction channel design, and developed in AZ405 (AZ Electronic Materials). Wet oxidation is then used to grow 100-μm-thick oxynitride layers at 900 °C for 10 h. After 20 min of baking at 90 °C, the wafer is etched with buffered oxide etch (BOE) and, using photolithography, a thin layer of photoresist is deposited to an oxide thickness of 300 nm and baked at 90 °C for 20 min. The photoresist is patterned using a chrome mask and developed in AZ405, and the oxide is then wet etched. The resulting oxide is then patterned using photolithography and deep reactive ion etching to form the etched channels and develop the device. Finally, the technique could potentially enable novel approaches to therapy (Fig. 6). One can envision an approach whereby a patient’s target cells are isolated from the blood or other tissue, treated by the device to deliver the desired therapeutic, and reintroduced into the body. Such an approach would take advantage of the potentially increased delivery efficiency of therapeutic macromolecules and could be safer than existing techniques because it would obviate the need for potentially toxic vector particles and would mitigate any potential side effects associated with reticuloendothelial clearance and off-target delivery.

**Conclusion**

In summary, we have detailed a method for cytosolic delivery that relies on the rapid mechanical deformation of a cell to induce transient membrane disruption. This technique has demonstrated the potential to deliver a broad range of materials, some of which are challenging to use with current methods, to a variety of difficult-to-transfect cell types, including stem cells and immune cells. By providing flexibility in application and obviating the need for exogenous materials or electrical fields, this method could potentially enable new avenues of disease research and treatment. Indeed, our work has demonstrated this system’s ability to deliver carbon nanotubes, quantum dots (38), and antibodies to live cells—applications that could enable new sensing and imaging modalities—and we have illustrated the system’s superior performance, relative to current methods, in applications such as transcription factor delivery for reprogramming.

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To deliver J Immunol Methods 168(2):422 cells (per sample) were treated 6 The home-built Raman system was used as previously | fl Nano Nat Rev Drug Discov vol. 110 19(5):506 | Confocal Microscopy. sured as the percentage reduction in a cell population | Collector from the outlet reservoir. Cells are incubated at room temperature | and placed in the device's inlet reservoir. This reservoir is connected to | a compressed air line controlled by a regulator, and the selected pressure (0–70 psi) is controlled by the fluid through the device. Treated cells are then collected from the outlet reservoir. Cells are incubated at room temperature in the delivery solution for 5–20 min after treatment to ensure hole closure before being subjected to any further treatment.

Delivery Materials. To deliver fluorescently labeled dextran molecules (Invitrogen), the experiments were conducted as described above such that the delivery buffer contained 0.1–0.3 mg/mL dextran. GFP knockdown is measured as the percentage reduction in a cell population's average fluorescence intensity relative to untreated controls. Lipofectamine 2000 plus siRNA was prepared by combining 1 μg of siRNA with 1 μL of Lipofectamine 2000 reagent in 100 μL of PBS.

Confocal Microscopy. Confocal images were taken using techniques described previously (38).


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