Microfluidic Viscoelastic Cell Squeezing for Intracellular Delivery

Nathan Raas*, Leon Krinn*, Kathryn Petersen*, Franz Görlich*

Inst. f. Chemie- u. Bioing.wiss., HCI F 117, Vladimir-Prelog-Weg 1-5/10,8093 Zürich, Switzerland

Supporting Information Placeholder

ABSTRACT: Transfection involves the intentional introduction of naked or purified nucleic acids into eukaryotic cells, offering potential cures for life-threatening diseases by genetic modification. Current transfection methods involve electroporation and lipofection but microfluidic approaches, notably "cell squeezing," present a novel and scalable technology. Here, we aim to optimize viSQZ, a versatile microfluidic intracellular delivery platform that leverages two immiscible fluids to squeeze cells. Results showed that while broader channels might sustain higher viabilities, narrower channels tended to achieve greater efficiencies in intracellular delivery. A trade-off between efficiency and viability was observed, emphasizing the need for a balance in channel geometry and flow ratios for optimal performance. viSQZ has several advantages over traditional approaches, including improved particle internalization, minimum cell alterations, inexpensive manufacturing, flexibility to different cell types and a clog-resistant design. Future research opportunities include testing different cell types, investigating alternative cargo, measuring real-time viability, simultaneous delivery of various macromolecules, and further chip design adjustments to enhance performance metrics. This study showcases the potential of microfluidic devices for efficient and adaptable intracellular delivery, paving the way for advancements in cell-based therapies.

Cell-based therapies are developing as the next generation of medicine, with the potential to prevent disease progression and cure a wide range of life-threatening disorders (1). To effectively transfer therapeutic cargos into cells, it is necessary to genetically modify the cells by delivering external cargos into the target cell. Transfection techniques such as electroporation, which uses high-voltage pulses to improve cell membrane permeability, are being utilized to transfer medicines, DNA, and other substances to the cell interior (2). Other methods include the use of a laser beam, gene injection, sonoporation, magnetofection, viral transfection, or the use of particular compounds to aid in transfection (3). Microfluidic techniques provide a novel route for intracellular delivery (4). A new intracellular delivery method called microfluidic "cell squeezing" has emerged as a relatively simple and scalable alternative (5). Cells are rapidly flowed through microfluidic tubes with narrow constrictions in this approach, boosting cell membrane permeability and allowing transfection as shown in Figure 1. Compared to traditional approaches, squeezing technologies require fewer cell alterations, are significantly cheaper and show improved particle internalization. The squeezing approach is very useful for intracellular delivery, but some limitations include channel blockage, delivery efficiency, and the need for an external electrical source. The aim of this project is to optimize the parameters of the microfluidic intracellular delivery technology device for different cargos. The device technology is referred to as viSQZ.

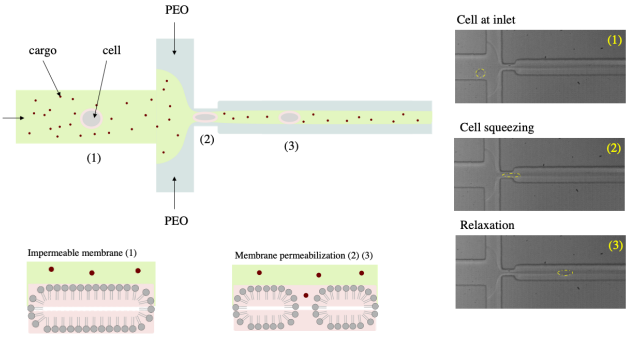

Figure 1. Operating principle of the viSQZ approach. Pores are generated in the cell membrane due to the squeeze induced by a narrow junction.

MATERIALS AND METHODS

Chip Fabrication. The PDMS based devices were fabricated according to standard soft-lithography procedures. Sylgard 184 Silicone elastomer (Dow Corning, USA) was mixed at a ratio of 10:1 base (dime-thylsiloxane, dimethylvinyl-terminated, and dimethylvinylated and trimethylated silica) to curing agent (Tetrakis(trimethylsilyloxy)silane). This mixture was poured onto a silicon wafer containing predetermined design structures with different channel lengths. The PDMS chips were then cured at 70°C for about 45 minutes. The structures were then removed from the master, cut, and hole-punched using a Gauge 20 hole-punch (Syneo, USA) to form the inlet and outlet ports. PDMS devices were then treated with plasma (Emitech K1000X, Quorum Technologies, UK) and sealed to a glass slide.

Parameter Optimization. To confirm that the technology indeed works according to the proposed hypothesis, 2000 kDa fluorescein isothiocyanate-conjugated dextran (FITC-dextran) was used as a cargo at a concentration of 0.3 µg/mL, and tested in a variety of squeezing lengths, and flow-rate ratios with K562 cells (ATCC CCL-243). The cell count was 10 million cells/mL. Squeezing lengths of 100, 300, and 500 µm were used with 600 kDa PEO in the sheath fluid at a constant PEO concentration of 0.1%, an optimized value based on previous work. For

each condition, sample to sheath flow ratios of 1:1, 1:2, 1:2.5 and 1:5 were tested using a sample flowrate of 5 µL/min and sheath fluid flowrates of 5 µL/min, 10 µL/min and 12.5 µL/min, respectively. To reach a ratio of 1:5, the sample flowrate was decreased to 2.5 µL/min and the PEO flowrate to 12.5 µL/min, to avoid channel bursting. After flowing through the microfluidic device, cells were collected, washed via centrifugation, resuspended in cell media, incubated for 24 h at 37 °C and 5% CO_2 and analysed using flow cytometry 24 to 72 hours later. To assess the potential of the viSQZ technology as a genomic editing tool, the same protocols were also carried out with a DNA plasmid at 50 µg/mL (pGreenPuro shRNA cloning and expresion Lentivirus, System Biosciences). The cargo was considered internalized if the emitted fluorescence was higher than the top 5% of the fluorescence measured in the control group as illustrated in Figure 4A (5). Thus, the efficiency is represented by the fraction of the live cells which showed a fluorescence above this treshold.

Data Processing, Analysis, and Visualization. To visualize the cells within the microfluidic channels, the microfluidic device was placed on an inverted Eclipse Ti-E microscope (Nikon, Zürich, Switzerland) with a high-speed camera (MIKROTRON, EoSens 3CL, Unterschleissheim, Germany). Bright field images were analysed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Microfluidic flow modelling was carried out in COMSOL Multiphysics® software (v. 6.2, COMSOL AB, Stockholm, Sweden). Flow cytometry data was analysed using FlowJo_v10.8.1 (BD Biosciences, Allschwil, Switzerland) software.

Principle of Operation. The creation of an artificial channel in which the cells travel is obtained by encasing the cell media by an immiscible sheet fluid through a cross junction. The width of the exiting end of the cross junction is narrower causing the thinning of the three phases and increasing their average velocity (see supplementary movies and COMSOL simulation). The designed system allows to control the width of the formed artificial channel in the gap, which causes to the formation of transient pores due to the squeezing of the cells, by varying the relative cell media and PEO flowrates. In the subsequent channel the width of both the PEO fluid fraction and the cell media fluid increase again, allowing cells to partially regain their initial volume and causing internalization of target molecules through the transient pores. The channel connection to the outlet occurs in two different configurations, denoted broad channel configuration and narrow channel configuration in the presented work (see Figure 2). In the broad channel configuration, the outlet is directly connected at the end of the channel whereas in the narrow channel configuration, a narrow passage before reaching the outlet is present which causes additional squeezing of the cells.

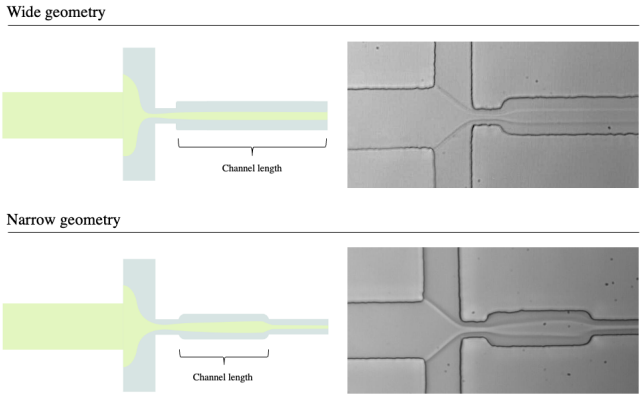

Figure 2. Wide/Borad geometry (top) and narrow geometry (bottom) used for transfection of cells.

Simulation. Numerical COMSOL simulations were performed to investigate the influence of different operating conditions on the virtual channel size. Note that the original goal of simulating a channel along with the cells did not work as the polymer flow module from COMSOL was not available. The channel was simulated using the laminar flow and two-phase flow module, assuming a high contact angle and low surface energy between water and PEO for describing the immiscibility. The results are presented in Figure 3 and show the expected behavior for the decrease of the virtual channel with increasing flow ratios.

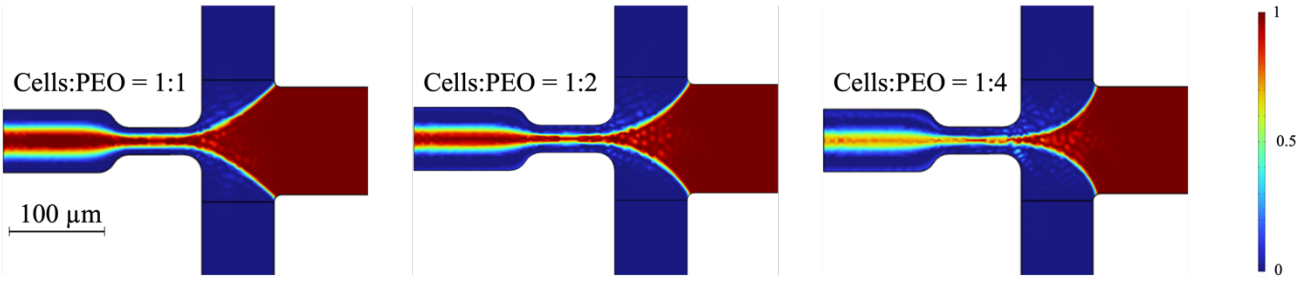

Figure 3. Volume fraction of sample after the stream is fully established inside the microfluidic channel for different flow ratios.

RESULTS AND DISCUSSION

Cell Viability and Internalization Efficiency in Broad Channels. Comparable cell viability was achieved when using the same ratios of cell media to PEO for channels with lengths of 100 µm and 300 µm. However, there was a noticeable decrease in cell viability for channels with a length of 500 µm. A typical fluorescence histogram for transfection of cells in the 100 µm broad channel is presented in Figure 4A while the viability for all borad channels is presented in Figure 4B and the efficiency in Figure 5B for different flow ratios and channel lengths. The fluoresence histograms for all tested conditions are provided in the supplementary information. After passing through the constriction in the virtual channel, deformation caused by squeezing occurred (see videos and COMSOL simulation). Extended periods of deformation have been found to have an adverse impact on cell viability, which is directly affected by the length of the channel (6). It was observed that, across all the channel lengths, the viability decreased as the PEO to cell media ratio increased. Even though a higher PEO ratio decreases the time it takes to deform the cells, an increase in the force exerted on the cells by the higher flowrate and the narrower virtual channel occurs. This phenomenon increases the occurance of cell death. Indeed, higher flowrates are reported to decrease cell viability in squeezing experiments (6). The decrease in viability observed when increasing the flowrate ratio from 1:2.5 to 1:5 was smaller than in the previous cases. This could be due to the reduced flowrate used at this ratio.

The vitality obtained at a 1:2.5 flow ratio was comparatively low, thus the cell media to PEO ratio of 1:5 was not tested with the 500 µm channel, as increasing the level of stress on the cells would lead to a decrease in performance. The efficiency augmented when the PEO ratio was increased from 1:1 to 1:2 but decreased at higher ratios. Efficiency of cargo internalization depends not only on the pores formed on the cells while being squeezed but also on relaxation time, local cargo concentration, and volume reincrease after squeezing (7). However, the precise relationship between the PEO ratio and these parameters in the presented system is not entirely clear.

At constant cell media to PEO ratios, similar efficiency values are obtained for the 100 µm and 300 µm channels, whereas a higher efficiency was found using the 500 µm channel indicating that longer channels lead to more efficient FITC-dextran internalization.

Cell Viability and Internalization Efficiency in Narrow Channels. In both the 100 µm and 300 µm narrow channels the viability was found to be independent of the PEO to cell media ratio (see Figure 4D). This result is unexpected because higher ratios cause more cell stress. Overall, higher viability values were obtained in the 100 µm channel compared to the 300 µm channel, indicating that viability decreases as the channel length increases. The 500 µm narrow channel was not tested

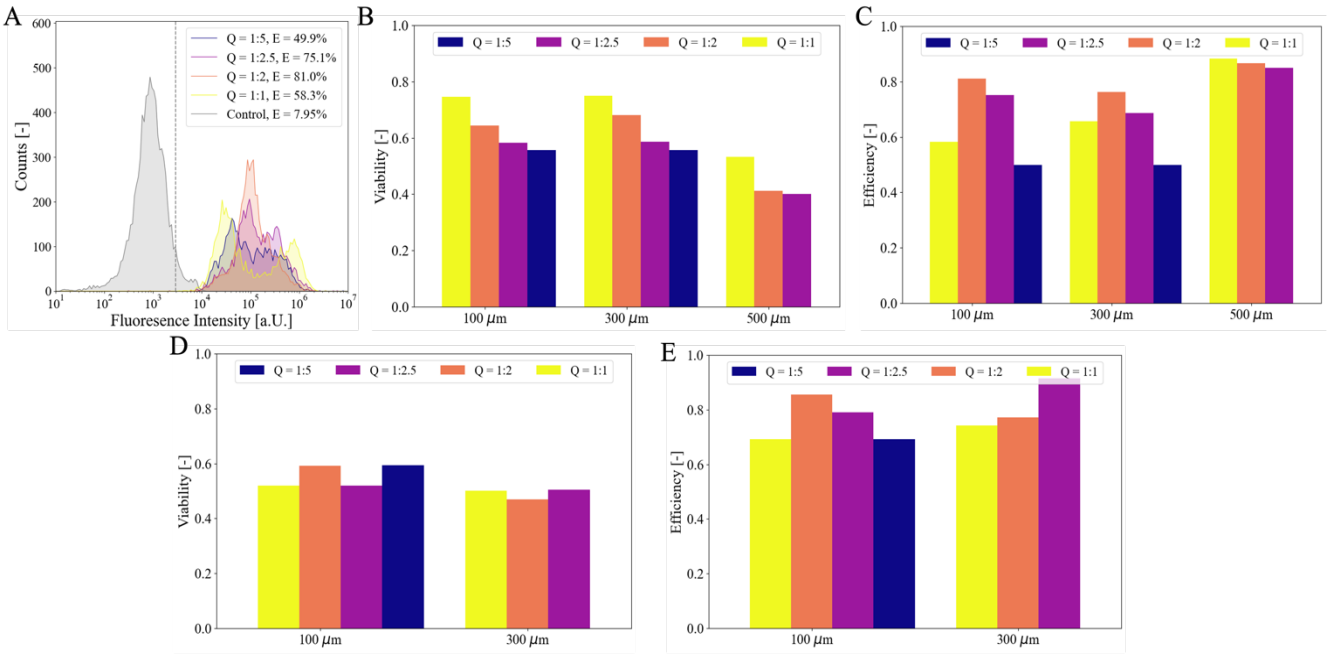

Figure 4. Dependence of flow rate and geometry on internalization of 2000 kDa FITC-dextran into K562 cells. (A) Fluoresence intensity histogram for transfection using the broad channel of 100 µm squeezing length, where Q denotes different flow ratios of sample to PEO and E the efficiency of internalization. (B) Viability and (C) efficiency for transfection using the broad geometry for different channel length and flow ratios Q. (D) Viability and efficiency (E) for internalization using the narrow channel geometry for different flow ratios Q and squeezing channel length.

since the obtained viability would be too low, thus not of interest.

In the 100 μm channel an increase in efficiency was observed when the cell media to PEO ratio was changed from 1:1 to 1:2, whereas it decreases again at higher ratios as shown in Figure 4E. Unexpectedly, for 300 μm the highest efficiency was found at 1:2.5 and not at 1:2 as in the broad channel. As previously cited, different parameters influence the efficiency and their dependency on the PEO flow rate could not be understood in the present experiments (6), (7). At the same cell to PEO ratios, higher efficiency values are obtained using the 300 μm channel compared to the 100 μm.

Configuration Comparison. As expected, all experiments lead to lower cell viability compared to the control group due to the high stress in the deformation process. The efficiency in any squeezing experiment was found to be significantly higher than the control, which had an efficiency of 7.95%. 2000 kDa dextran is hardly internalized into the cell by diffusion alone due to its large size, leading to low efficiency values in the control group (6). A six-fold increase in the efficiency proves the efficacy of the method and indicates internalization of dextran in both configurations due to convective flows generated during the deformation (6).

Both the narrow and the broad channel configurations show similar trends in terms of efficiency changes with respect to channel length and cell media to PEO ratios. The increase of the PEO ratio from 1:1 to 1:2 resulted in a higher efficiency, but efficiency decreased at higher ratios. This behaviour was not observed in the case of the 500 μm broad channel and 300 μm narrow channel. In the former, efficiency decreased as the PEO to cell media ratio increased, whereas in the latter the efficiency continued to increase at a 1:2.5 ratio. At constant channel lengths and PEO ratios the narrow channel achieved a higher efficiency compared to the broad channel due to the decrease in cross section at the end of the channel, which causes an additional squeezing of the cells (6).

Similar viability values are obtained for broad 100 μm and 300 μm channels, whereas it decreased at 500 μm. In the case of the narrow channel configuration, viability was found to decrease when changing the channel length from 100 μm to 300 μm. Moreover, viability was not dependent on the PEO ratio in the tested interval. As expected, lower viability values are obtained at the same length and ratios when the narrow channel is used due to the additional squeezing of the cells.

Channel Selection for Optimal Performance. The highest efficiency recorded was 91.5% and was obtained using a 300 μm narrow channel at a 1:2.5 cell media to PEO ratio whereas the highest cell viability was 75.0% and was found using a 300 μm broad channel at a 1:1 ratio. However, these values are linked to either poor efficiency or poor viability. Thus, there is a trade-off between efficiency and viability. A performance metric is obtained by calculating the fraction of live cells that were successfully inoculated with respect to the total number of cells, obtained by multiplying the measured efficiencies and the viabilities (since the former represents the fraction of live cells that were inoculated and the latter the fraction of cells that survived) (5). The 300 μm and 100 μm broad channels at a cell media to PEO ratio of 1:2 achieved the highest number of living and successfully inoculated cells with values of 52.2% and 51.8%, respectively. An acceptable performance (50.5%) is also obtained using a 100 μm narrow channel with a ratio of 1:2.

Plasmids Internalization Performance. Based on the previous results, the broad channel configuration with a channel length of 100 μm and operated at a cell media to PEO ratio of 1:2 was chosen to carry out plasmid internalization, since it delivered the highest performance using 2000 kDa FITC-dextran. The obtained transfection efficiency and cell viability after an incubation time of 72 hours were 88.6% and 78.9%, respectively. The designed system delivers both high viability and efficiency, leading to a performance of 64.4%. The results prove the effectiveness of the viSQZ technology for the internalization of hard-to-transfect particles such as plasmids. Moreover, the system was found to outperform other microfluidics approaches such as droplet squeezing and conventional transfection methods like lipofection and electroporation (6).

CONCLUSIONS

Results indicate varying effects on cell viability and efficiency based on squeezing lengths, flow ratios, and channel configurations. Broad channels exhibit viability oscillations with PEO ratios, while narrow channels show unexpected efficiency patterns at different ratios. Notably, the 300 μm narrow channel at a 1:2.5 ratio demonstrated the highest efficiency (91.5%), while the 300 μm broad channel at a 1:1 ratio achieved the highest cell viability (75.0%). The 100 μm broad channel at a 1:2 ratio yielded the highest performance. Practical application for transfection using a plasmid encoding a GFP protein was demonstrated to be successful with a viability of 78.9% and a efficiency of 88.6% using a 100 um broad channel at a 1:2 ratio.

Compared to other approaches, devices deforming cells by means of narrow channel sections, are reported to be near clog-free (6). However, in the presented device, cell deformation occurs *via* compression due to the PEO flow, so large debris are less likely to clog the channels making this configuration even more efficient compared to the state of the art. Moreover, this device is easily adapted to different cell types since the PEO and cell media flow rates can be tuned to ensure best performance in terms of viability and efficiency. The ability to control the virtual channel size allows adaption of the

chip to cells of different dimensions without the need to resort to a new design.

Future Works. Future work includes the testing of different cells types to assess the chip performance with cells of different dimensions and deformability, the evaluation of the average working time before clogging, the internalization of different macromolecules beyond the ones tested, (e.g. mRNA, FITC-dextran with different sizes, nanomaterials or therapeutic agents) and modifications of the chip design to achieve better performances in terms of viability and efficiency. Moreover, in the presented method cell viability was tested only after an incubation period. Modifying the chip to include a real time assessment of cell viability would be beneficial, as it would ensure that excessive damage is not caused to the cells. A similar approach could also be developed to observe real time delivery. In the present work only one type of macromolecule was internalized at a time, thus the performance of the method could be assessed also in the case of simultaneous delivery of different macromolecules.

ASSOCIATED CONTENT

Supporting Information. Figures of fluorescence histograms of all tested conditions as well as for the plasmid internalisation are provided in the file SupportingInformationSqueeze.docx.

AUTHOR INFORMATION

Corresponding Authors

Nathan Raas – ETH, Zurich, Switzerland, raasn@ethz.ch
Leon Krinn – ETH, Zurich, Switzerland, lkrinn@ethz.ch
Kathryn Petersen – ETH, Zurich Switzerland, kpetersen@ethz.ch
Franz Görlich – ETH, Zurich, Switzerland fgoerlich@ethz.ch

ACKNOWLEDGMENT

We would like to thank Prof. DeMello and Rashin Mohammadi for supervision and guidance. We also would like to thank Thomas Moragues for the help with simulatioms in COMSOL.

REFERENCES

1. *Engineering the next generation of cell-based therapeutics.* **Bashor, Caleb J., et al.** 9, s.l. : Nature Research, 9 2022, Nature Reviews Drug Discovery, Vol. 21, p. 655-675. 14741784.

2. *Electroporation: Theory and methods, perspectives for drug delivery, gene therapy and research.* **Gehl, J.** 4, 4 2003, Acta Physiologica Scandinavica, Vol. 177, p. 437-447. 00016772.

3. *Transfection types, methods and strategies: A technical review.* **Chong, Zhi Xiong, Yeap, Swee Keong e Ho, Wan Yong.** s.l. : PeerJ Inc., 4 2021, PeerJ, Vol. 9. 21678359.

4. *Massively-Parallelized, Deterministic Mechanoporation for Intracellular Delivery.* **Dixit, Harish G., et al.** 2, s.l. : American Chemical Society, 2 2020, Nano Letters, Vol. 20, p. 860-687. 15306992.

5. *A vector-free microfluidic platform for intracellular delivery.* **Sharei, Armon, et al.** 6, 2 2013, Vol. 110, p. 2082-2087. 00278424.

6. *Highly Efficient Transfection of Human Primary T Lymphocytes Using Droplet-Enabled Mechanoporation.* **Joo, Byeongju, et al.** 8, s.l. : American Chemical Society, 8 2021, ACS Nano, Vol. 15, p. 12888-128987. 1936086X.

7. *Microfluidic generation of transient cell volume exchange for convectively driven intracellular delivery of large macromolecules.* **Liu, Anna, et al.** 7, 2018 : Elsevier B.V., 9, Materials Today, Vol. 21. 18734103.

8. *Microfluidic cell stretching for highly effective gene delivery into hard-to-transfect primary cells.* **Hur, Jeongsoo, et al.** 11, s.l. : American Chemical Society, 11 2020, ACS Nano, Vol. 14. 1936086X.