Supplementary information

Nanoinjection System for Precise Direct Delivery of Biomolecules into Single Cells

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Fabrication of microfluidic device

The photomask film containing microchannel pattern of PDMS layer was designed using the VCarvePro CAD software (Vectric Ltd., Redditch, UK). A SU-8 negative photoresist was coated by a spin coater (ACE-200, Dong AH trade Corp., Seoul, Korea) on the base Si wafer and patterned to make a mold using conventional photo-lithography (Fig. S1a). Then, a PDMS base and a curing agent (SYLGARD® 184 Silicone Elastomer Kit, Dow corning Corp., Midland, MI, USA) were vigorously mixed with a 10:1 ratio, and the glutinous mixture was subsequently poured onto the mold and cured at 70 °C for 8 h in an incubator (Fig. S1b). The polymerized PDMS was peeled off from silicon masters mold. Then, fluidic connections to an outside line were achieved by machining holes with a 1-mm diameter biopsy punch (Kai medical Inc., Honolulu, HI, USA) (Fig. S1c). The PDMS was bonded on top of the glass substrate by O_2 plasma bonding (CUTE, Femto science, Suwon, Korea) for 1 min (Fig. S1d). It was named the "header chip". Prior to nanoinjection structure fabrication, the microchannel of header chip was washed three times with distilled water. Fig. S1e shows the schematic illustration of the nanoinjection structure machined by using femtosecond-laser (fs-laser) in the bottom glass layer of the header chip.

fs-laser Machining system configuration

The header chip was placed on a computer-controlled X-Y-Z axis (100 μm × 100 μm × 100 μm) piezo-stage (Nano-LP100, Mad city labs Inc., Madison, WI, USA) for 3-D nano-machining. The nanoinjection structure inside the glass layer (Fig. S2) was formed using the fs-laser machining system (Femtobiomed, Seongnam, Korea) described in detail elsewhere[1,2]. The laser pulse, whose wavelength was 513 nm (50 mW) wavelength with a frequency of 2 kHz, was used for glass processing (Fig. S3).

SEM imaging

To evaluate the influence of the fs-laser melting parameters in the glass layer, different parameters were evaluated: Spindle speed (laser intensity, μm), feed rate (laser speed, μm/min), start depth (laser melting point, μm). The morphology of the fs-laser induced glass surface modifications was analyzed

by a Scanning Electron Microscope (SEM) (Fig. S4). For this purpose, the sample was coated with a Pt layer using a Cressington 208 HR sputter coater under an argon atmosphere. The coated samples were examined and imaged by a JEOL JSM-7401F at an accelerating voltage of 5.0 kV. SEM images analysis revealed the optimal combination of parameters at value of spindle speed, feed rate, start depth which are 8000 rpm, 480 μm/min, 0 μm-Z axis, respectively, and the nanoinjection structure was fabricated under optimal parameters.

Channel Resistance of nanoinjection system

The electrolyte conductivity (σ) and resistivity (ρ) are the parameters connected with the purity of the water. The electrical conductivities of the electrolytes were measured using a conductivity meter (TI9000, Trans instruments Ltd., Singapore). Electrolyte conductivity is expressed as microsimens per centimeter (μS/cm). Resistance (ρ) is the reciprocal of conductivity and is expressed as the ohm-meter (Ωm). When voltage is applied to the aqueous solution, the electric current is generated by the flow of the ions in the solution; therefore, the electric current flows abundantly if many ions are present, while the conductivity is high if the resistance is low. Moreover, conductivity and resistance are influenced by the temperature. This research proceeded in a laboratory that was maintained at room temperature.

The conductivity of the solution was measured according to the electric current passing through the electrode that was connected to the solution-filled microtube. The D-PBS conductivity, represented as $D\text{-}PBS_{\sigma}$, was then calculated using the following equation (1):

$$\sigma\,[S/m] = \frac{L\,[m]}{R\,[\Omega] \times A[m^2]} \quad (1)$$

where R is the microtube resistance, L is the length of the channel, and A is the cross-sectional area of the microtube.

To measure the channel resistance, the D-PBS solution was filled in the header chip channel where the nanoinjection structure is included. The flow resistance of all the channels in the header chip is approximated by the following equation (2):

$$R\ [\Omega] = \frac{\rho\ [\Omega m^2/m]\ \times\ L\ [m]}{A[m^2]} \quad (2)$$

Also, we can obtain an expression for L[m]/A[m^2] in the geometries of the PDMS microchannel using the measured values of the oscilloscope resistance, applied voltage, and D-PBS conductivity.

The microchannel resistance of a nanoinjection structure is easily calculable using the equation (2). The nanochannel size of the nanoinjection structure was influenced by the laser intensity because of the fs-laser focal-spot size. therefore, the pore diameter of the nanochannels is adjustable with the use of the laser intensity. Here, we fabricated nanochannel with diameters of 800 nm. The resistivity of all the channels is marked on Table S1.

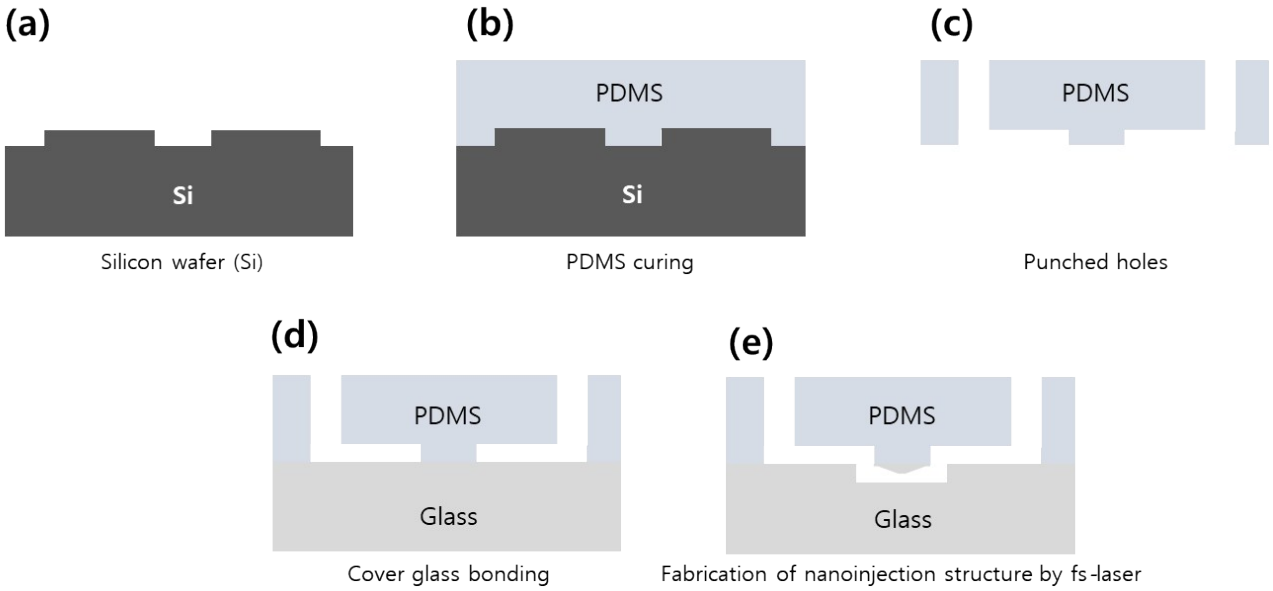
Cell viability

We confirmed the viability of cells before and after processing the cells on the nanoinjection system (CellShot™ platform, supplier: Femtobiomed Inc.). After electrical stimulation, the collected cells were stained using Calcein-AM (Sigma-Aldrich, St. Louis, MO, USA) used to label living cells. 2 μM calcein-AM were incubated with the sample for 30 min at room temperature. The phase difference and fluorescence images of the cells were obtained for image processing.

Reference

1 S. Lee, *Univ. Mich.*, 2008, **Doctor of Philosophy**, 128.

2 S. Lee, R. An and A. J. Hunt, *Nat. Nanotechnol.*, 2010, **5**, 412–416.



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106 **Fig. S1** Fabrication process of header chip and nanoinjection structure. The soft lithography
107 techniques are combined with the laminated object manufacturing techniques. (a-c) Transferring the
108 pattern from a Si wafer onto the PDMS mold. (d) Pre-mold with microchannel structure of a header
109 chip was bonded to the cover glass. (e) Nanoinjection structure machined by using the fs-laser in the
110 bottom glass layer.

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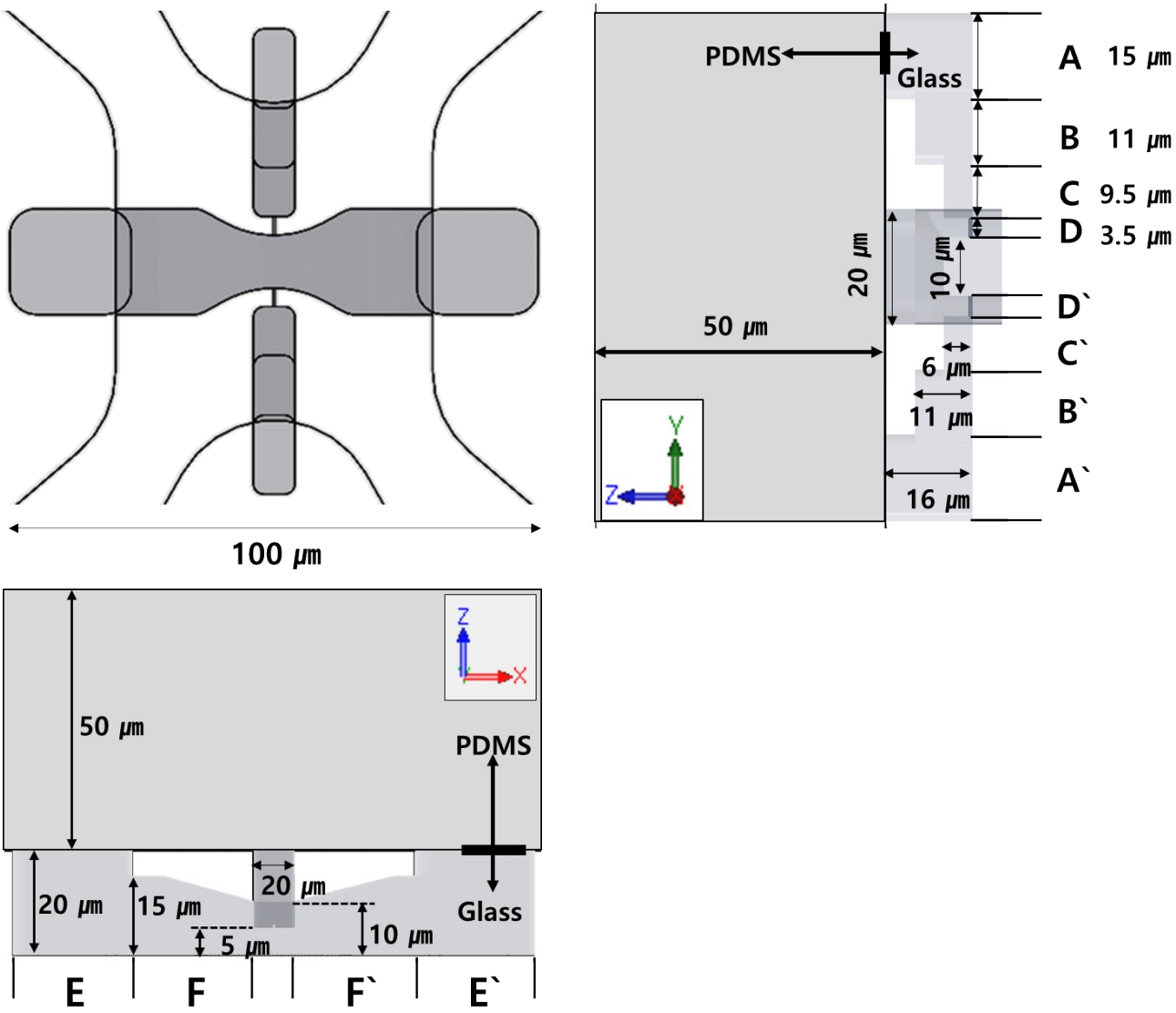

Fig. S2 Standard specification of nanoinjection structure. A-C, A`-C`: charge (+, -) channel; D, D`: Nanochannel; E-F, E`-F`: Cell-loading channel.

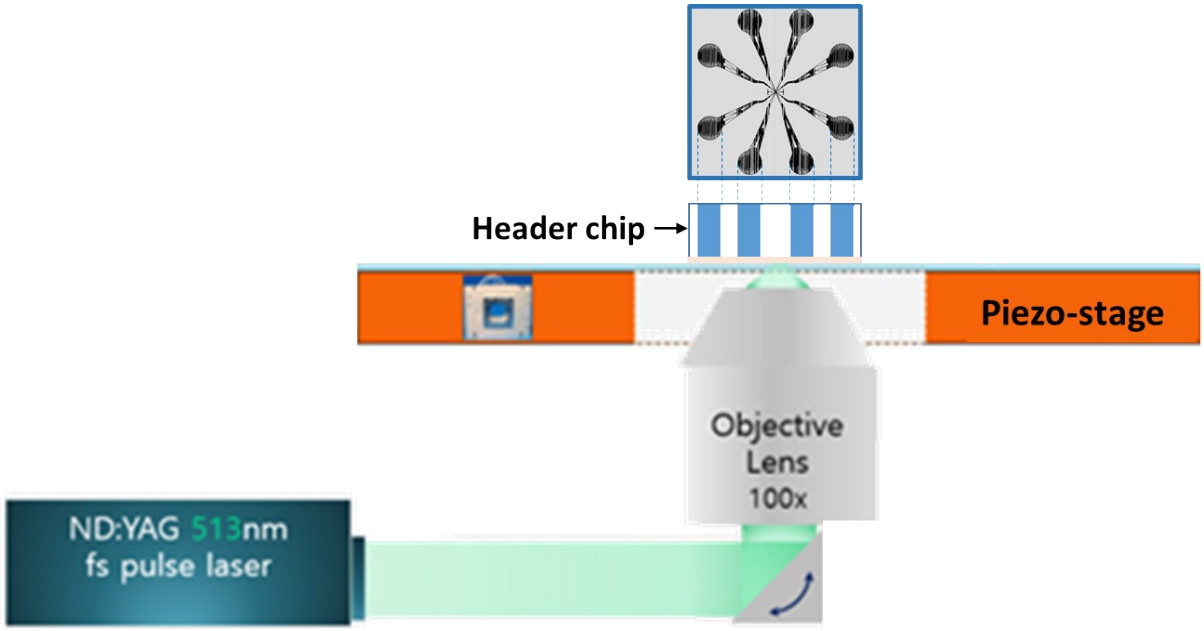

Fig. S3 Schematic diagram of a fs-laser machining system for fabrication of nanoinjection structure.

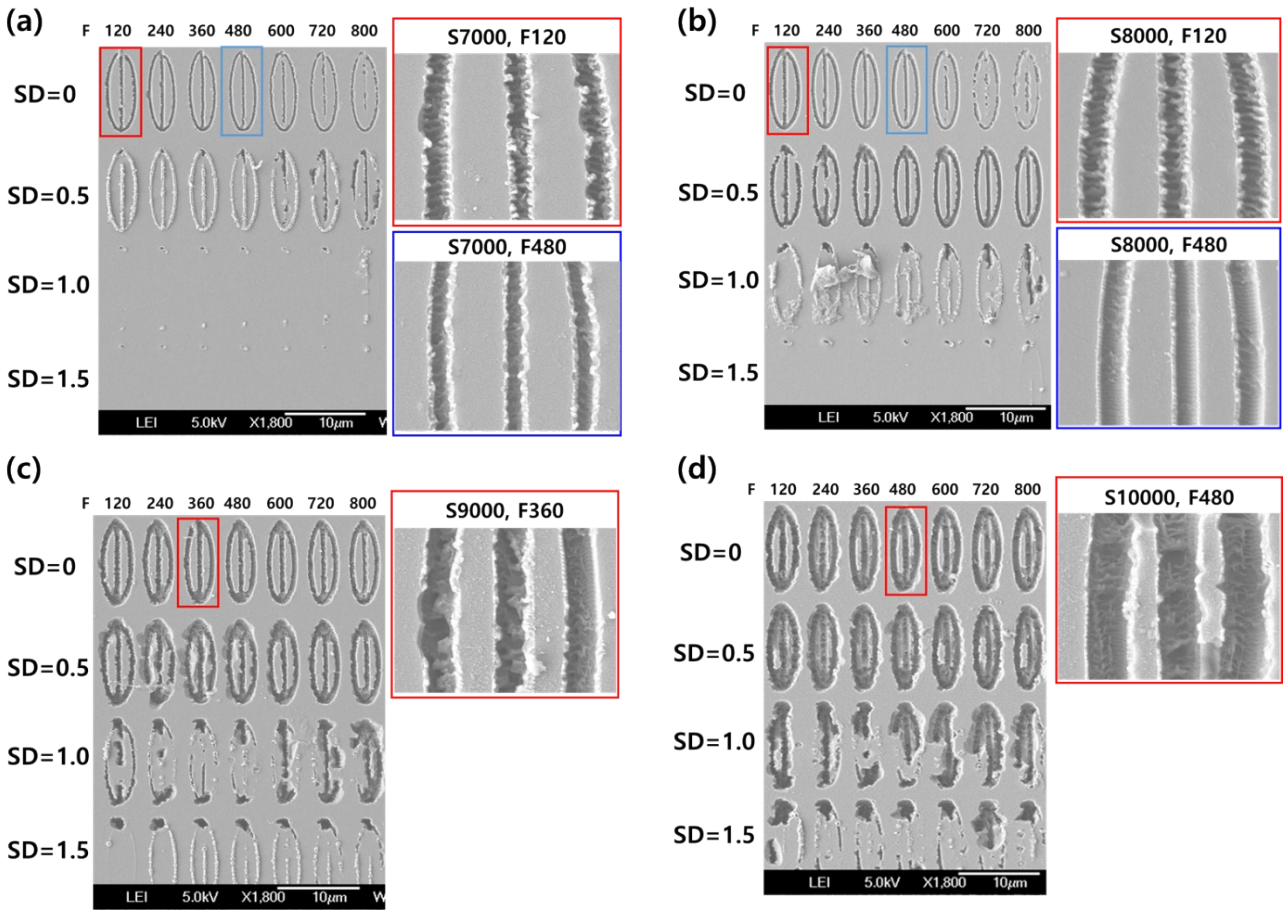

Fig. S4 SEM images of line-ellipse patterns formed using fs-laser. (a-d) are SEM images prepared at Spindle speed (S, laser intensity) of 7000, 8000, 9000, and 10000 rpm, respectively. The tests were carried out at feed rate (F, laser speed) up to 800 μm/min and start depth (SD, laser melting point) up to 1.5 μm-Z axis.

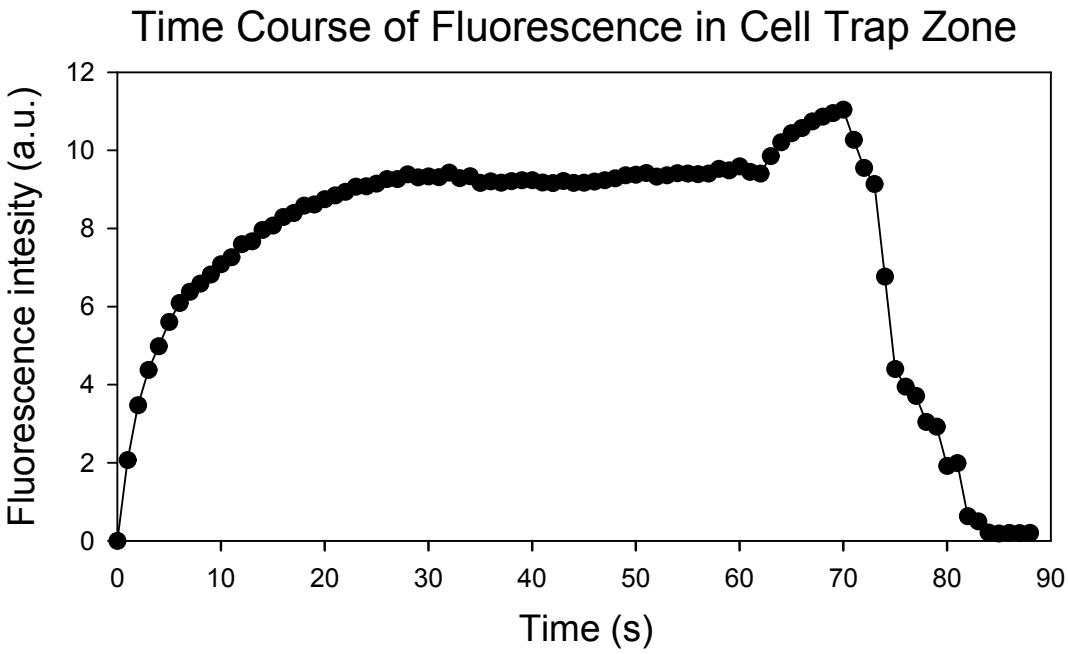

Fig. S5 RFP fluorescence intensity inside the cells separated by pulse duration.

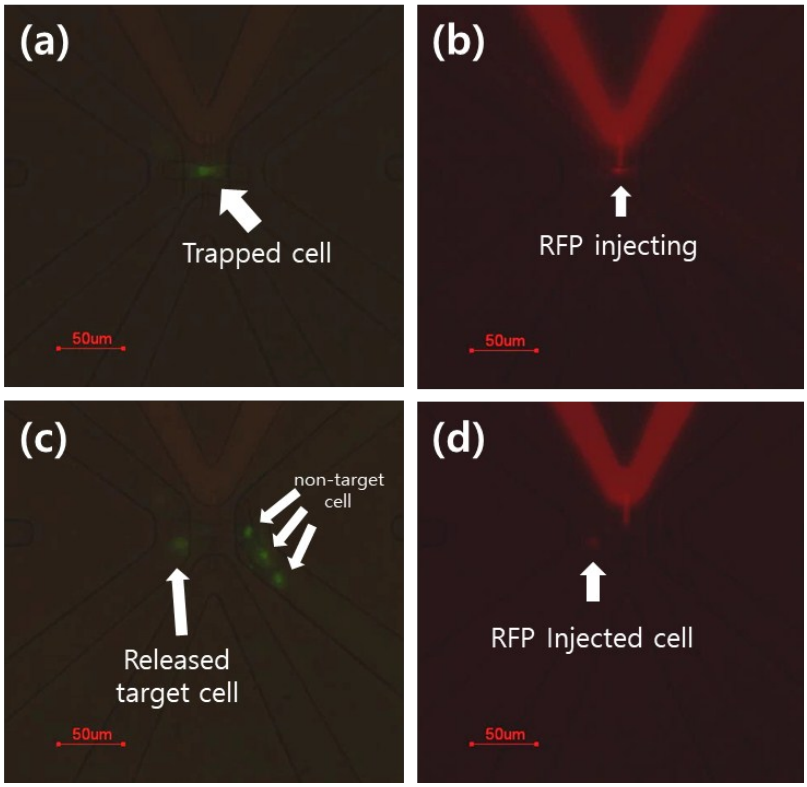

Fig. S6 Fluorescence image of RFP-injected into a human umbilical cord-derived stem cells (hUC-MSC). (a) Trapped cell (Green: CalceinAM), (b) RFP-injected cell, (c) Comparison of target cell and non-target cells with fluorescence (Green: CalceinAM), (d) Comparison of target cell and non-target cells with fluorescence (Red: RFP).

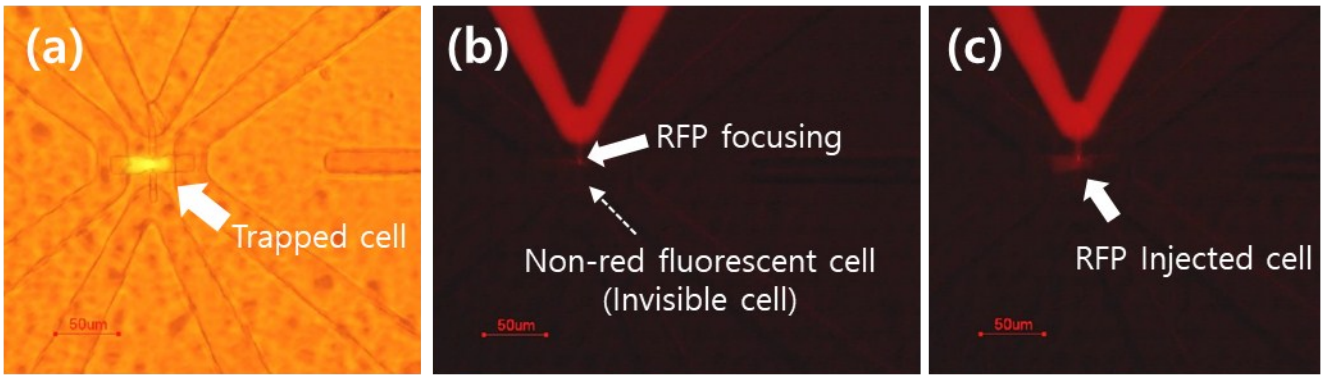

Fig. S7 Fluorescence image of RFP-injected into a A549 cell. (a) Trapped cell (Green: CalceinAM), (b) Non-red fluorescent trapped cell, (c) RFP-injected cell (Red: RFP).

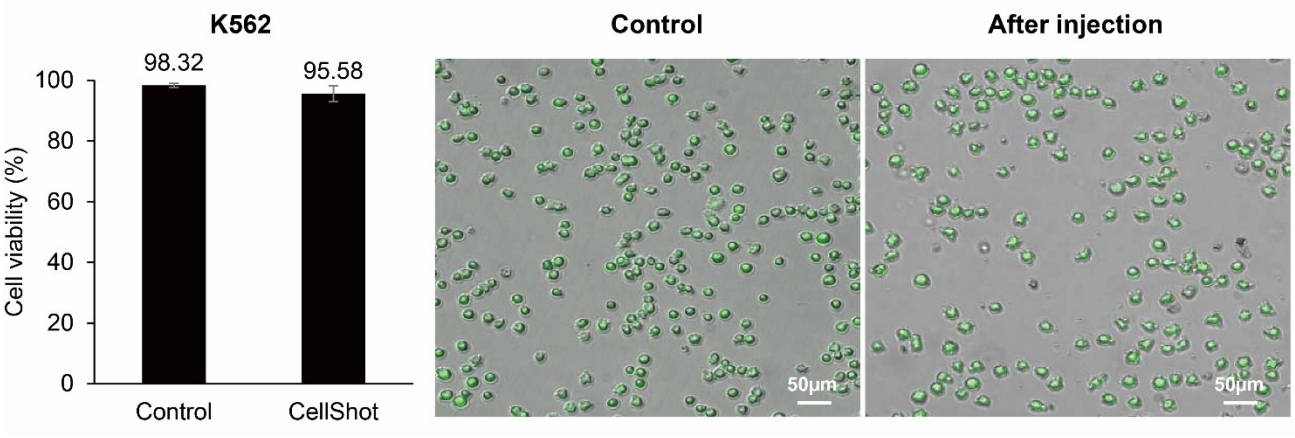

Fig. S8 Quantitative image analysis of cell viability based on immunofluorescence intensity. The cell viability after treated in nanoinjection system is >95% for K562 cells (human immortalized myelogenous leukemia cell line). The cell viability was confirmed by Calcein-AM staining. The data were expressed as mean ± standard error (# of live cells / #of collected cells).

Supplementary table

Channel			Resistance (ohm, Ω)	Sub. Total (ohm, Ω)	Voltage (V)
Positive (+) charge channel inlet	PDMS channel		645,484	645,484	0.13
	Top microchannel	A	85,470	306,268	0.06
		B	85,470		
		C	135,328		
	Top nanochannel	D	4,642,019	4,642,019	1.0
Negative (-) charge channel inlet	Bottom nanochannel	D`	4,642,019	4,642,019	1.0
	Bottom microchannel	C`	135,328	158,333	0.06
		B`	85,470		
		A`	85,470		
	PDMS channel		645,484	710,960	0.13
Oscilloscope			1,000,000	1,000,000	0.21
Total				7,349,154	2.5

Table S1 Nano and microchannel resistance of nanoinjection structure. The distribution of the applied voltage of 2.5 V of an integrated electrode on a nanoinjection system.

Supplementary movie

Movie S1 RFP fluorophore flow without cell.

Movie S2 RFP fluorescence intensity inside the cells by pulse duration.

Movie S3 Destruction of living cell in nanoinjection system by high-voltage application.

Movie S4 Continuous intracellular delivery process.