Electronic Supplementary Information

Title

A new microfluidic platform for the highly reproducible preparation of non-viral gene delivery complexes

Author list

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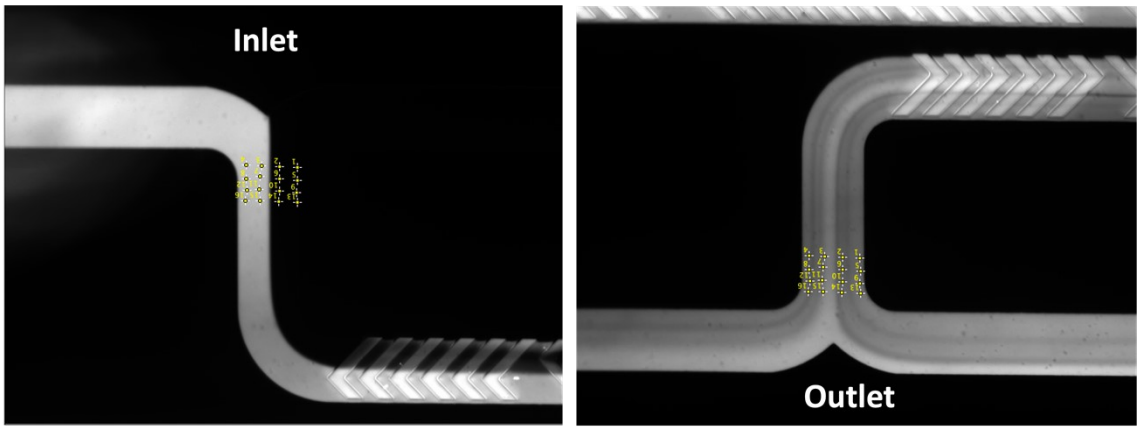
Table of contents

Figure S1. Digital images of the first level of the SDG. Yellow crosses represent the regions of interest (ROIs) used for the fluorescence intensity measurements.



1.1 Design of the chaotic serial dilution generator (SDG)

The SDG was designed to generate a linear gradient of polymer concentrations (from 100 % to 0 % with steps of 16.7 %), according to the symmetrical model described by Jeon *et al.*,[1] and provided with two inlet and seven outlet ports (**Figure S2A**). The underlying mechanism of mixing and splitting of the resulting polymer solutions flowing within the cartridge is described through the hydraulic circuit represented in **Figure S2B**.

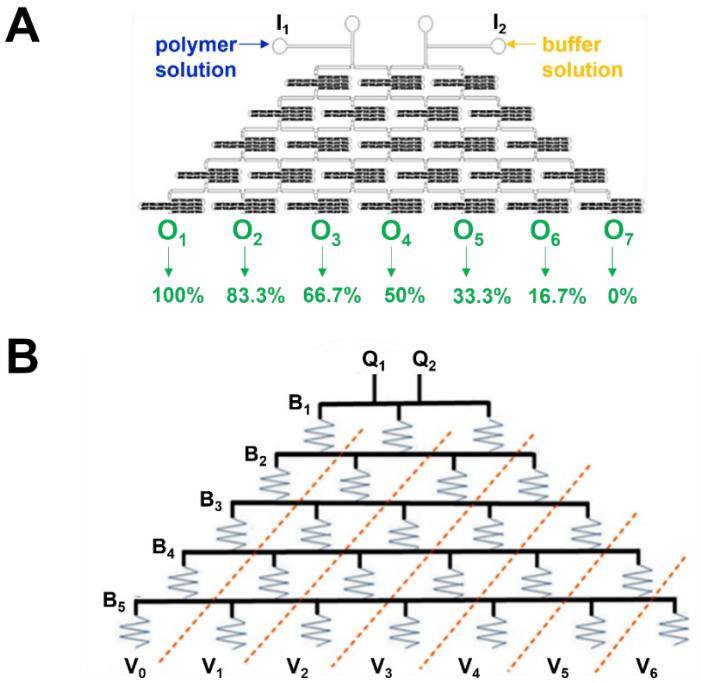


Figure S2. Serial dilution generator (SDG). (A) The two main inlets (I_1 and I_2, blue and yellow arrows) and the seven outlet ports (O_1-O_7, green arrows) are highlighted. (B) Equivalent electric circuit model of the pyramidal microfluidic network. Q_1 and Q_2: flow rates of the polymer solution and the buffer injected into I_1 and I_2, respectively. B_1-B_5: branches at each of the 5 SDG levels; V_0-V_6: vertical channels of the branched system.

Briefly, according to the Hagen-Poiseuille's law (eq. 1):

$$\Delta P = R_h \times Q \quad \text{(eq. 1)}$$

where:

- ΔP is the pressure drop at the extremities of a channel;
- Q [$\frac{m^3}{s}$] is the flow rate within the channel;
- R_h [$\frac{Pa\,s}{m^3}$] is the hydraulic resistance of the channel.

Using its electrical equivalent (governed by the Ohm's law: $\Delta V = R \times I$), the voltage drop ΔV $[V]$ between two points in a conductor is proportional to the current I $[A]$ flowing between them, through an electrical resistance R $[\Omega]$). The hydraulic resistance of each channel was calculated assuming a rectangular-shaped microchannel with a low aspect ratio (i.e., width ≈ height), as follows (eq. 2):

$$R_h = \frac{12\,\mu L}{wh^3}\left[1 - \frac{h}{w}\left(\frac{192}{\pi^5}\sum_{n=1,3,5}^{\infty}\frac{1}{n^5}\tanh\left(\frac{n\pi w}{2h}\right)\right)\right]^{-1} \quad \text{(eq. 2)}$$

where:

- μ [Pa·s] is the dynamic viscosity of the fluid;

- *L* [m] is the channel length;
- *w* and h [m] are the channel width and height, respectively.

The flow rates Q_1 and Q_2 of the solutions at I_1 and I_2 were set as equal and the resistances of the horizontal channels were neglected, as their length was much shorter than the vertical channels length.
The flow distribution in the microfluidic chip was calculated as follows (eq. 3-4):

$$\text{Left flow fraction} = \frac{B - V}{B + 1} \quad \text{(eq. 3)}$$

$$\text{Right flow fraction} = \frac{V + 1}{B + 1} \quad \text{(eq. 4)}$$

For symmetry reasons, the flow rate in every vertical channel (Q_v) downstream the SDG was given by (eq. 5):

$$Qv = (\frac{1}{7} \times (Q1 + Q2)) \quad \text{(eq. 5)}$$

In order to ensure the proper mixing of the two solutions with the microfluidic cartridge, the minimum channel length (L_D) was calculated as a function of the diffusion coefficient of the input substance, the geometry of the channel and the flow rate, as follows (eq. 6):

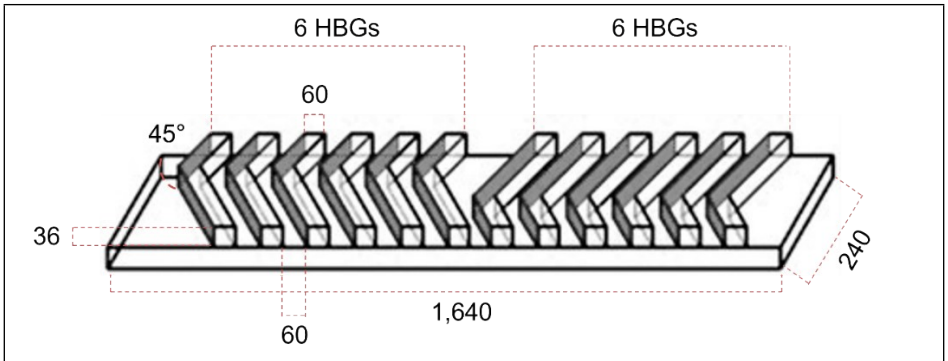
$$L_D = U \times t_D = u \times d^2/D_D = Q/D_D \quad \text{(eq. 6)}$$

where:
- *U* $[m/s]$ is the fluid velocity;
- t_D $[s]$ the diffusion time;
- *d* $[m]$ is the diffusional length;
- D_D $[m^2/s]$ is the diffusion coefficient;
- *Q* $[m^3/s]$ is the flow rate.

1.2 Design of the staggered herringbone grooves (HBGs)

The ceiling of each device channel was fitted with staggered herringbone grooves (HBGs) to ensure an effective chaotic mixing. The HBGs were designed to be as wide as the main fluidic channels (240 µm), 60 µm-long and 36 µm-high, and orientated at a 45° angle (y-angle) with respect to the long axis of the channel. Each single HBG unit is composed of a rectangular 1,640 µm-long channel, integrated with 12 HBGs, reversing their orientation every six HBGs (see **Table S1**).

Table S1. HBG unit dimension (µm).



HBG height [µm]	36
HBG length [µm]	60
HBG width [µm]	240
HBG y-angle [°]	45

HBG number in a HBG unit	6 + 6
HBG unit length [µm]	1,640
Distance between HBGs [µm]	60

In order to ensure the proper mixing of the polymer and generate a linear gradient of polymer concentrations, the minimum number of repeating HBG units was estimated according to the following equation[2] (eq. 7):

$$L_{min} \sim \lambda \times ln(Pe) \quad \text{(eq. 7)}$$

where:

- L_{min} is the minimum length of the HBG unit;
- λ is the length determined by the geometry of trajectories in the chaotic flow;
- *Pe* is the Peclet number, calculated as follow (eq. 8):

$$Pe = \frac{v \times l}{D} \quad \text{(eq. 8)}$$

where:

- v is the velocity of the solution in the channel, calculated as $\frac{Q}{A}$ (where Q is the incoming flow rate at each SDG level, A is the cross-section area calculated as follows $A = w \times h$, where w and h are, respectively, the width (240 µm) and the height (100 µm) of the channel);
- l is the dimension of the channel (set equal to 100 µm);
- D is the diffusion coefficient, calculated by using the Stokes-Einstein relationship (eq. 9):

$$D = \frac{kT}{6\pi\mu r} \quad \text{(eq. 9)}$$

where:

- k = 1.38 ×10^{-23} (J × K^{-1}) is the Boltzmann's constant;
- T = 298.15 K is the room temperature (r.t.);
- μ = 8.94 × 10^{-4} (Pa·× s) is the viscosity;
- r is the mean radius of the polymer, estimated as $\sqrt[3]{M_w}$, where M_w is the molecular weight of the polymer.

1.3 Design of the NA divider and the polymer-DNA mixing units

The NA divider (**Figure S3A**) was designed to split and distribute the same volume of a starting DNA stock solution to the seven polymer/DNA mixing units. To do this, the NA divider was equipped with an inlet (I_3) connected to a resistive flow pattern of seven microfluidic channels, connected to the seven SDG outlet ports. As for the SDG, each polymer/DNA mixing unit (**Figure S3B**) was equipped with HBGs to ensure the proper mix of the upstream polymer dilutions from the SDG with the DNA solutions from the NA divider.

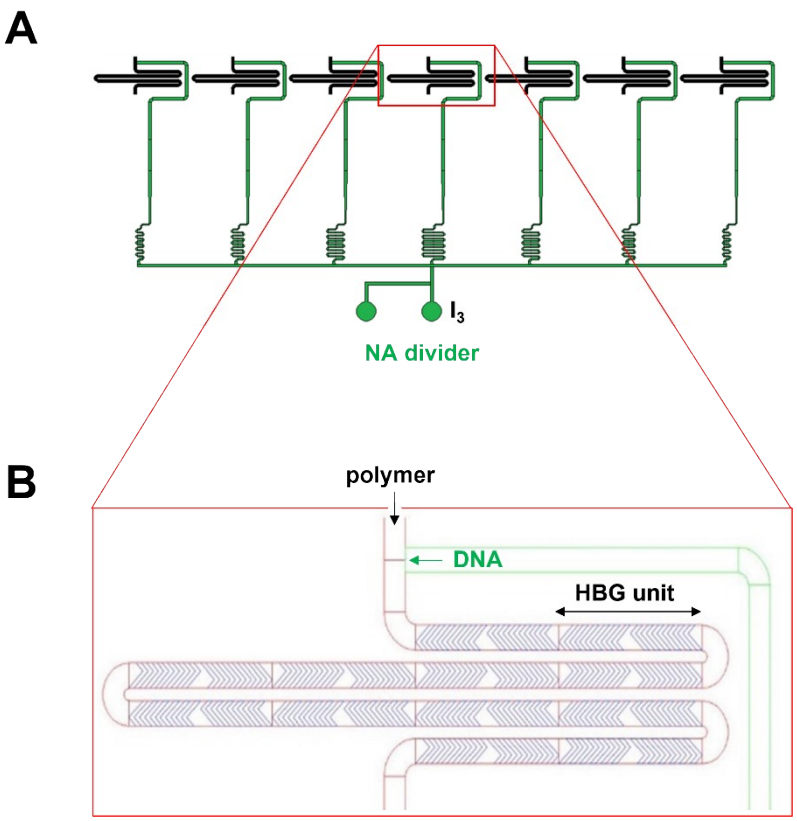


Figure S3. Layout of the downstream section of the device. (A) Schematic of the NA divider. The main inlet I_3 and the seven DNA channels are highlighted. (B) Magnified view of a polymer/DNA mixing unit, encompassing 12 HBG units integrated within the device microchannels.

1.4 Manual preparation of PEI/pDNA complexes at a single N/P

Polyplexes were invariably prepared at r.t. by adding (1:1 (v/v)) the DNA solution (0.25 μg/μL in 0.1× TE buffer) to the transfectant solution at a given concentration to yield polyplexes at the desired N/P. Polyplexes were allowed to form for 20 min and used right after preparation. pGLuc and pGL3 plasmids were utilized to prepare polyplexes for transfection assays (DNA dose: 320 μg/cm^2, corresponding to 100 ng DNA/well), while salmon sperm DNA (ssDNA; 1 μg/μL in 0.1× TE buffer) was used for physicochemical characterization purposes.

Figure S4. DNA distribution through the NA divider.

The device was perfused with a SYBR Green I-labelled DNA solution from the inlet 3 (I_3) at a flow rate of 50 µL/min, while the upstream channels of the SDG were filled through I_1 and I_2 with 10 mM HEPES (flow rates = 25 µL/min). The fluorescence intensity of solutions collected through the O_1-O_7 ports was measured. Data were expressed as fluorescence intensity normalized to their mean value, which was taken as a reference (n = 3).

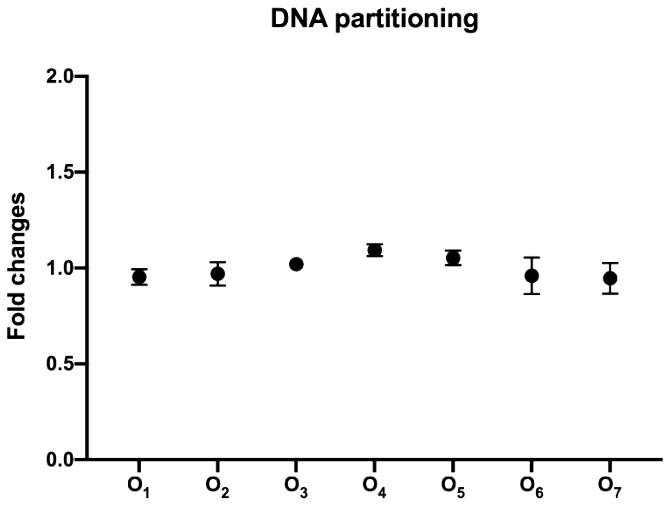


Figure S5. Comparative cytotoxicity and transfection efficiencies of polyplexes prepared at N/P 40 using the microfluidic device and by manual pipetting.

A) Cytotoxicity and (B) transfection efficiency of polyplexes prepared by mixing pGLuc and 25 kDa /PEI at N/P 40 with the microfluidic device (*operation mode 2*) (black, empty boxes) and by manual pipetting (red striped boxes). Results on L929 cells are displayed as box and whiskers plots (n ≥ 3). *p < 0.05, ***p < 0.001 *vs.* manual pipetting (in Test for Two Variances).

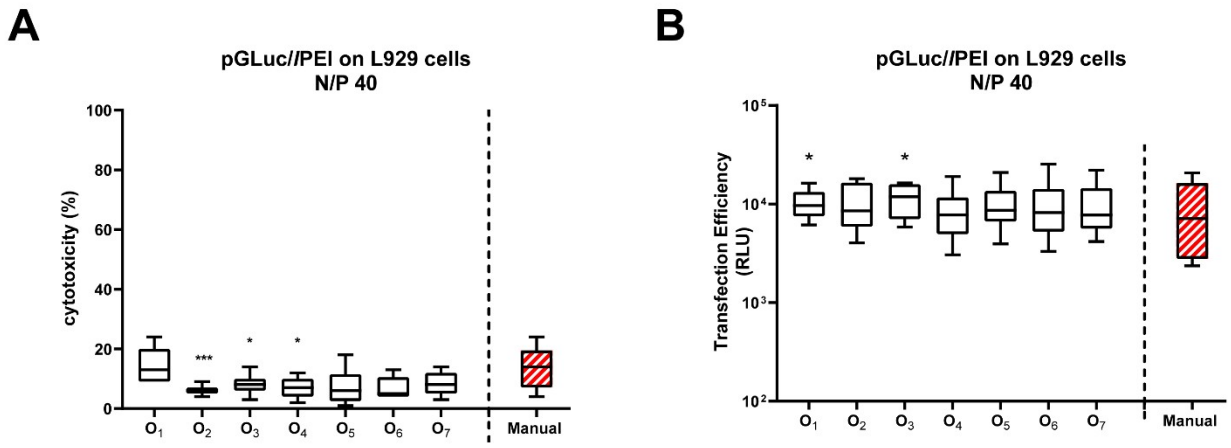


Figure S6. Comparative cytotoxicity and transfection efficiencies of polyplexes prepared at different N/Ps using the microfluidic device and by manual pipetting.

A) Cytotoxicity and (B) transfection efficiency of polyplexes prepared with the microfluidic mixing (*operation mode 1*) (black boxes) and by manual pipetting (red boxes), mixing variable amounts of branched PEI (*b*PEI) with a constant pGL3 content in 10 mM HEPES buffer to give different N/Ps (10, 20, 30, 40, 50, and 60). N/P 0 means no transfection reagent. Results on Jurkat cells are displayed as box and whiskers plots ($n \geq 3$). *$p < 0.05$, ***$p < 0.001$ between microfluidic and manual preparations (in Test for Two Variances).

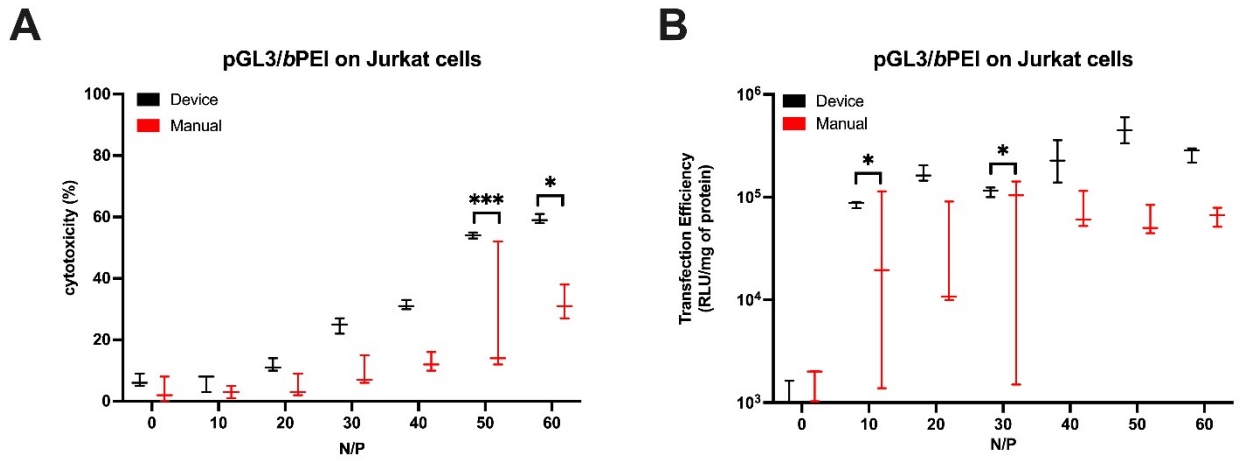


Table S2. Length of each mixing unit upstream of the outlet ports.

mixing unit 1 [mm]	mixing unit 2 [mm]	mixing unit 3 [mm]	mixing unit 4 [mm]	mixing unit 5 [mm]	mixing unit 6 [mm]	mixing unit 7 [mm]
25.13	25.01	24.95	25.09	25.10	25.09	24.91

Table S3. Hydrodynamic diameter (DH), polydispersity index (PDI), and ζ-potential (ζ_P) of polyplexes at varying N/Ps (operation mode 1) measured with Dynamic Light Scattering (DLS) and Laser Doppler micro-electrophoresis.

25 kDa *l*PEI-based polyplexes were prepared at varying N/Ps (*operation mode 1*) in 10 mM HEPES at pH 7 through the addition of ssDNA to the PEI solution. The polymer and the NAs were mixed with the device (microfluidic preparation) or by pipetting (manual preparation). Measurements were performed 5 min after dilution in buffer and expressed as mean ± standard deviation (n = 3).

Transfection reagent	Buffer	N/P	Preparation	D_H (nm)	PDI	ζ_P (mV)
25 kDa /PEI	10 mM Hepes	10	MICROFLUIDIC	165 ± 18	0.6 ± 0.10	19 ± 3
		10	MANUAL	151 ± 9	0.6 ± 0.30	4 ± 2
		20	MICROFLUIDIC	204 ± 7	0.2 ± 0.16	25 ± 6
		20	MANUAL	135 ± 6	0.7 ± 0.30	10 ± 1
		30	MICROFLUIDIC	118 ± 26	0.2 ± 0.07	23 ± 3
		30	MANUAL	137 ± 6	0.6 ± 0.10	11 ± 3
		40	MICROFLUIDIC	145 ± 10	0.2 ± 0.08	29 ± 3
		40	MANUAL	176 ± 54	0.5 ± 0.10	7 ± 3
		50	MICROFLUIDIC	98 ± 7	0.1 ± 0.03	27 ± 4
		50	MANUAL	196 ± 10	0.4 ± 0.20	8 ± 4
		60	MICROFLUIDIC	126 ± 45	0.4 ± 0.10	22 ± 3
		60	MANUAL	158 ± 17	0.5 ± 0.20	7 ± 2

Table S4. Hydrodynamic diameter (D_H), polydispersity index (PDI), and ζ-potential (ζ_P) of polyplexes at N/P 40 measured with Dynamic Light Scattering (DLS) and Laser Doppler micro-electrophoresis.

25 kDa *l*PEI-based polyplexes were invariably prepared at N/P 40 (*operation mode 2*) in 10 mM HEPES at pH 7 through the addition of ssDNA to the PEI solution. The polymer and the NAs were mixed through the device (microfluidic preparation). Measurements were performed 5 min after dilution in buffer and expressed as mean ± standard deviation (n = 3).

Transfection reagent	Buffer	Preparation	Outlet	D_H (nm)	PDI	ζ_P (mV)
25 kDa *l*PEI	**10 mM Hepes**	MICROFLUIDIC	O_1	140 ± 2	0.1 ± 0.01	34 ± 1.2
			O_2	161 ± 12	0.2 ± 0.09	31 ± 1.9
			O_3	151 ± 42	0.3 ± 0.01	32 ± 0.9
			O_4	142 ± 20	0.2 ± 0.10	32 ± 0.4
			O_5	135 ± 3	0.1 ± 0.01	31 ± 0.8
			O_6	155 ± 33	0.3 ± 0.05	29 ± 2.5
			O_7	134 ± 9	0.2 ± 0.04	27 ± 2.7

References

1 N. L. Jeon, S. K. W. Dertinger, D. T. Chiu, I. S. Choi, A. D. Stroock and G. M. Whitesides, *Langmuir*, 2000, **16**, 8311–8316.

2 A. D. Stroock, S. K. W. Dertinger, A. Ajdari, I. Mezić, H. A. Stone and G. M. Whitesides, *Science.*, 2002, **295**, 647–651.