ADVANCED HEALTHCARE MATERIALS

Supporting Information

for *Adv. Healthcare Mater.*, DOI: 10.1002/adhm.202100125

Influence of the modulation of the protein corona on gene expression using polyethylenimine (PEI) polyplexes as delivery vehicle

Dingcheng Zhu, Huijie Yan, Zhuxian Zhou, Jianbin Tang, Xiangrui Liu, Raimo Hartmann, Wolfgang J. Parak, Youqing Shen*, Neus Feliu*

Influence of the modulation of the protein corona on gene expression using polyethylenimine (PEI) polyplexes as delivery vehicle

Dingcheng Zhu[1,2,3], Huijie Yan[1,2], Zhuxian Zhou[1], Jianbin Tang[1], Xiangrui Liu[1], Raimo Hartmann[4], Wolfgang J. Parak[2,5], Youqing Shen[1*], Neus Feliu[2,6*]

[1]Zhejiang Key Laboratory of Smart Biomaterials and Key Laboratory of Biomass Chemical Engineering of Ministry of Education, College of Chemical and Biological Engineering, Zhejiang University,Zheda road 38, Hangzhou, 310007, China
[2]FachbereichPhysik und Chemie and CHyN, Universität Hamburg, Notkestraße 85, Hamburg, 22607, Germany
[3] College of Material, Chemistry and Chemical Engineering, Hangzhou Normal University, Haishu road 58, Hangzhou, 310000, China
[4] FachbereichPhysik, Philipps Universität Marburg, Renthof 6, Marburg, 35032, Germany
[5] CIC biomaGUNE, MiramonPasealekua 182, San Sebastian, 20014, Spain
[6] Fraunhofer Center for Applied Nanotechnology (CAN), Grindelallee 117, Hamburg, 20146, Germany
[*] corresponding authors: shenyq@zju.edu.cn, nfeliu@physnet.uni-hamburg.de

Supporting Information

I) Reagents

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; #H3375), bovine serum albumin (BSA, #V900933), fluorescein isothiocyanate conjugated BSA (BSA^{FITC}, #A9771), branched polyethyleniminewith a molecular weight of 25 kDa (PEI, #408727),andrhodamine B isothiocyanate(#283924-100MG), Hoechst 33342 (#B2261)were purchased from Sigma-Aldrich. pGL4.13 [*luc2*/SV40] luciferase-encoding plasmid (pLuci, #TM259) was purchased from Promega. Enhanced green fluorescent protein (eGFP)-encoding plasmid (peGFP-N1) was kindly provided bythe School of Medicine, Zhejiang University[1]. Red fluorescent protein-encoding plasmid was kindly provided by the Shanghai Institute of Materia Medica[2]. All plasmids were propagated in Escherichia coli DH5α and were extracted using the Endo-Free Plasmid Giga Kit (#12362, Qiagen). The LabelIT®Tracer™ Intracellular Nucleic Acid Localization Kit, $Cy5^{TM5}$ (#7021, Mirus) was purchased from Mirus Bio. The luciferase-encoding plasmid was labelled with Cy5 (DNA^{Cy5}) as reported previously[1a].All chemicals were used as received. Milli-Q water with a resistance greater than 18.2 MΩ cm^{-1} was used for all experiments.

II) Synthesis of materials

II.1) Rhodamine B isothiocyanatelabeling of PEI

To label PEI with rhodamine B isothiocyanate(PEI^{RBITC}), PEI (10.5 mg, 238.6μmolof the repeat unit) was dissolved in 600μL of 0.1 M $NaHCO_3$ buffer. Rhodamine B isothiocyanate (1 mg, 2.3μmol) dissolved in 50 μL of dimethyl sulfoxide (DMSO) was added, and the mixture was incubated overnight and protected from light. Unreacted dye was removed by PD-10 desalting columns (#17-0851-01, GE Healthcare) usingMilli-Q water as the eluent. The concentration of purified PEI was measured via the copper(II) complexation method[3],and the concentration of the conjugated dye was calculated according to Beer–Lambert's law.The molar absorbance coefficient ε of rhodamine B at its absorption peakis 80,000 $M^{-1}cm^{-1}$. The experimentally determined labelling density was 0.055μmolrhodamine B/mg PEI.

II.2) Polyplex formation

PEI was dissolved in 10 mM HEPES-NaOH aqueous solution (pH 7.4) at a mass concentration of C_{PEI} = 40μg/mL, and the solution was filtrated before use. Plasmid DNA was diluted in 10 mM HEPES-NaOH buffer(pH 7.4) to prepare a mass concentration of C_{DNA} = 40 μg/mL. PEI/DNA polyplexes (i.e., $P_{1/1/0}$) were prepared by adding PEI solutions into an equal volume of DNA solution, and the mixture was immediately vortexed for 5 seconds. Afterwards, polyplexes were left undisturbed at room temperature for 20 min before use.

For PEI/DNA coated with BSA at a PEI/DNA/BSA weight ratio of 1/1/4 (i.e., $P_{1/1/4}$), BSA wasdissolved in 10 mM HEPES-NaOH aqueous solution (pH 7.4) at a mass concentration of C_{BSA} = 1600 μg/mL and was filtrated before use. One volume of BSA solution was added into two volumes of $P_{1/1/0}$ solution, and the mixture was immediately vortexed for 5 s. Afterwards, $P_{1/1/4}$ were left undisturbed at room temperature for 20 min before use.

III)Polyplex delivery and gene expression

III.1) Cell culture

Human cervical carcinoma HeLa cells (#93021013, Sigma Aldrich, European Collection of Authenticated Cell Cultures) were cultured in Dulbecco's Modified Eagles Medium (DMEM, #11965092, Thermo Fisher Scientific) with 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany, #S0615), 1% penicillin/streptomycin (P/S, #15070063, Thermo Fisher Scientific) at 5% CO_2 and 37°C.

III.2) Uptake studies by flow cytometry

HeLa cells were seeded in 24-well plates at 6.4×10^4 cells per well (1.9 cm^2 seeding area per well) in 800 µL of cell culture medium containing 10% FBS and were incubated overnight to reach 70~80% cell confluence. On the next day, cells were incubated with 800 µL of fresh serum free culture medium containing $P_{1/1/0}$ or$P_{1/1/4}$ polyplexes at 1.4 µg/mL DNA (200 µg/mLDNA^{Cy5}and 1.2 µg/mL unlabeled DNA) for t = 0.5, 1, 2, 4, and 6 h. Afterwards, the medium was removed, and cells were gently washed with phosphate buffered saline (PBS), detached by trypsin, isolated, resuspended in PBS, and analyzed immediately using flow cytometry.The excitation was at 640 nm and the recording of the emission was done at 670 nm with a 30 nm bandpass. 10,000 gated cells were counted. Cellular association in terms of mean Cy5 fluorescence intensity per cell were analyzed by the software FlowJoV10. Cells without exposure to polyplexes were used as control.

III.3) Gene transfection

For transfection using pLcui,HeLa cells were seeded in 48-well plates at 3.2×10^4 cells per well (0.95 cm^2 seeding area per well) in 400 µL of cell culture medium containing 10% FBS andwere incubated overnight to reach 70~80% cell confluence. To study the influence of the incubation time, cells were incubated with $P_{1/1/0}$ and $P_{1/1/4}$ polyplexes at 1.4 µg/mL DNA for t = 0.5, 1, 2, 4, or 6 h in 400 µL of serum free cell culture medium, followed by t' = 24 h culture in fresh cell culture medium containing 10% FBS without polyplexes. To study the influence of transfection time, cells were incubated with $P_{1/1/0}$ and $P_{1/1/4}$ polyplexes for t = 3 h in 400 µL of serum free cell culture medium, followed by t' = 3, 6, 9, 12, 18, and 24 h culture in fresh cell culture medium containing 10% FBS without polyplexes. The luciferase expression was measured in terms of luminescence intensity I_{Luci}, which was evaluated by an illuminator usingthe Luciferase Assay System (#E1500,Promega). The protein content of the cell lysate was measured by the Bradford protein assay kit (#C505031, Sangon Biotech). Luminescence was normalized by the amount of

protein as RLU/mg. Experiments were repeated three times (n = 3).

For peGFP and pRFP mediated transfection, HeLa cells were seeded on µ-slide 8 well plates (#80824, Ibidi, 1.0 cm^2 seeding area per well) at2.4×10^4 cells per well in 300µL of culture medium one day prior to use. On the next day, cells were incubated with 300 µL of fresh serum free cell culture medium containing$P_{1/1/0}$ or$P_{1/1/4}$ polyplexes at 1.4 µg/mL DNA for t = 3 h, followed by t' = 24 h culture in fresh cell culture medium containing 10% FBS. To calculate the percentages of cells expressing eGFP, RFP or both, cells were stained with 15 µg/mL of Hoechst 33342 in PBS for 15 min and were imaged by confocal laser scanning microscope (CLSM) at 10 × magnification. The data were analyzed by the software Cellprofiler (v 3.1.8) (see **§IV.1**). Experiments were repeated three times (n = 3).The data shown in **Figure 2** in the manuscript were imaged at 63 × magnification for better resolution, and nuclei staining by Hoechst 33342 was not conducted in this case.

To check the effect of fluorescence labeling of PEI (i.e.,PEI^{RBITC}) and peGFP (i.e., DNA^{Cy5}) on transfection, $P_{1/1/0}$ prepared as PEI/DNA, PEI^{RBITC}/DNA, PEI/DNA^{Cy5}, and PEI^{RBITC}/DNA^{Cy5} were used. HeLa cells were seeded in 24-well plates at 6.4×10^4 cells per well (1.9 cm^2 seeding area per well) in 800 µL of cell culture medium containing 10% FBS and were incubated overnight to reach 70~80% cell confluence. On the next day, cells were incubated with $P_{1/1/0}$ with different labeling at 1.4 µg/mL DNA for t = 3 h in 800 µL of serum free cell culture medium, followed by t' = 24 h culture in fresh cell culture medium containing 10% FBS without polyplexes. The percentage of cells expressing eGFP was determined by flow cytometry and the data are shown in **Figure SI.III.3.1**. More details about these experiments can be found in our previous publication [1a].

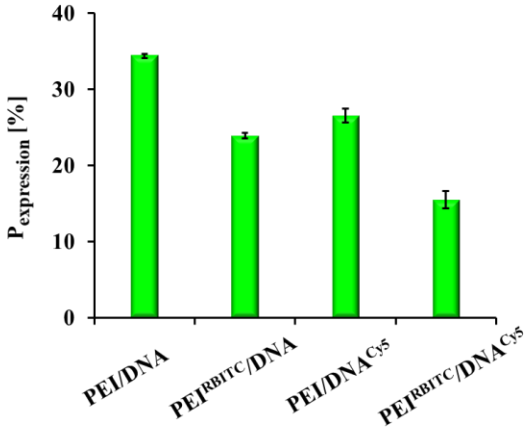

Figure SI.III.3.1 Effect of fluorescence labeled PEI and peGFP on the transfection of $P_{1/1/0}$ polyplexes.Four polyplexes with different labeling were prepared as PEI/DNA, PEI^{RBITC}/DNA, PEI/DNA^{Cy5}, and PEI^{RBITC}/DNA^{Cy5}. Cells were incubated with polyplexes at 1.4 µg/mL DNA in serum free culture medium for t = 3 h, followed by further culture in fresh cell culture medium containing 10% FB for t' = 24 h. The cells were analyzed by a flow cytometry, and the fraction

of fluorescent cells was calculated. The transfection efficiency $P_{expression}$ is thus given in terms of percentage of cells expressing eGFP.

III.4) mRNA transcription by real time polymerase chain reaction (RT-PCR)

HeLa cells were seeded at a density of 1.6×10^5 cells per well in 6-well plates (9.5 cm^2 seeding area per well)in 2 mL of cell culture medium containing 10% FBS and were incubated overnight. Cells were incubated with 2 mL of serum free cell culture medium containing polyplexes at 1.4 µg/mLpLuciin for t = 3 h. Afterward, cells were further incubated for 2, 4, 8, 12, 18, and 24 h in 2 mL of fresh cell culture medium containing 10% FBS. Cells were washed with PBS for 2 times, and RNA was extracted using the RNAiso Plus Kit (#9109, TaKaRa). RNA was diluted with autoclaved diethyl pyrocarbonate (DEPC) aqueous solution (0.1% w/v) to 100 ng/mL andwas reversed transcribed to cDNA using the Prime Script™ RT Reagent Kit (#RR037B, TaKaRa). Note that RNA is highly unstable, and it can be degraded by even trace amounts of RNase. RNase may be still partly active even after autoclaving. Usually, DEPC is dissolved in water to deactivate RNase. But DEPC itself is highly toxic and carcinogenic. However, it can be decomposed by auto-claving. Therefore, DEPC containing water was used to deactivate RNase and then autoclaved to remove DEPC. The cDNA was quantified by RT-PCR using theSYBR®PremixExTaq™ II kit (#RR820A, TaKaRa) ina StepOne Real-Time PCR System (Life technologies). Thesequences of primers were usedas follows: Luci-F: 5'-CTAAGGTGGTGGACTTGGACAC-3'; Luci-R: 3'-CGATGAGAGCGTTTGTAGCC-5'; GAPDH-F: 5'-AGAAGGCTGGGGCTCATTTG-3'; GAPDH-R: 3'-AGGGGCCATCCACAGTCTTC-5'. GAPDH was utilized as the endogenous gene control. The samples were pre-incubated at 95 °Cfor 30 s. Subsequently, 40 cycles were conducted: denaturation at 95 °C for 5 s and extension at 60 °C for 30 s. The results were analyzed with the StepOne Software v2.3. Experiments were repeated three times (n = 3).

III.5) Cell proliferation

The proliferation of HeLa cells was measured by the methyl blue method[4]. Briefly, cells were seeded in 24-well plates (1.9 cm^2 seeding area per well) at a density of 6.4×10^4 cells per well in 800 µL of cell culture medium containing 10% FBS. After 6, 12, 19, 23, 30, 44, and 50 h culture, the medium was removed, and cells were washed with PBS for 2 times. To mimic transfection conditions, themedium was removed at 23 h and cells were incubated with fresh FBS free culture medium for 3 h. At 26 h, the medium was replaced with fresh culture medium containing 10% FBS. Cells were washed with PBS for 2 times, fixed with formalin (10%v/v) in PBS for 10 min, washed with PBS for 2 times,and incubated with 400 µL of methyl blue aqueous solution (0.5 wt % in PBS) at 37℃ for 1 h. Unfixed dye was washed awayby PBSfor 5 times and fixed

dye was extracted by incubating cells with 600 µL of elution solution containing 50% ethanol, 49% PBS, and 1 % acetic acid for 15 min. The absorbance was measured at a wavelength of 662 nm. Experiments were repeated for three times (n = 3). The data are shown in **Figure SI.III.5.1.**

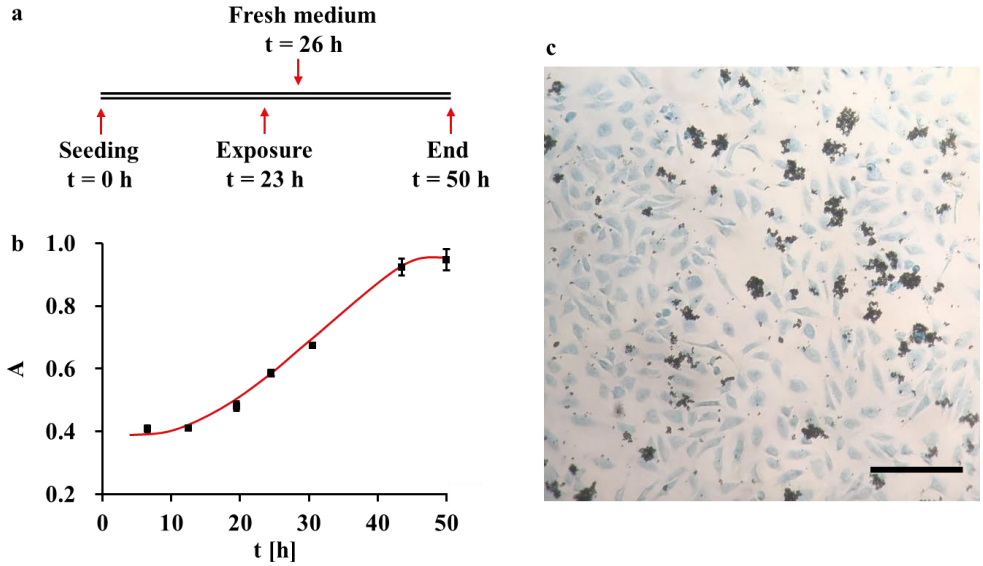

Figure SI.III.5.1. Proliferation of HeLa cells. a) The experimental timeline for measuring cell proliferation. Cells were seeded at t = 0 h and incubated overnight. At 23 h, cells were incubated in fresh serum free cell culture medium.At 26 h the medium was replaced with fresh cell culture medium containing 10% FBS. The measurement ended at 50 h. b) Proliferation curve of HeLa cells. After 6, 12, 19, 24, 30, 44, and 50 h culture, cell densities in terms of absorbance of methyl blue were measured. c) Representative image of cells after stained with methyl blue (shown in blue). The scale bar represents 200 µm.

III.6) Cell imaging by confocallaser scanning microscope (CLSM)

Fluorescence imageswere acquiredby CLSM (510 Meta, Zeiss). Appropriate laser excitation and filter sets wereused for the different fluorophores (Hoechst 33342: wavelength of excitation λ_{ex} = 405 nm;the emission was recorded with a bandpass (BP) fromλ_{em} 420-480 nm; eGFP and FITC: λ_{ex} = 488 nm and λ_{em}= 505-550 nm; RhodamineB and RFP: λ_{ex} = 543 nm, λ_{em}= 560-615 nm; Cy5: λ_{ex} = 633 nm, λ_{em}= 650-750 nm). The laser power,pinhole, and gains were optimized to avoid over-exposure. All figures are representative of entire fields. The brightness, contrast, excitation laser power, and pinholes were all set to the same values for all images in the same group.

For live imaging of gene expression, HeLa cells were seeded on µ-slide 8 well plates (1.0 cm^2 seeding area per well) at 2.4×10^4 cells per well in 300µL of culture medium one day prior to use. On the next day, cells were incubated with 300 µL of fresh serum free cell culture mediumcontaining $P_{1/1/0}$ or$P_{1/1/4}$ polyplexes at 1.4 µg/mLpeGFPfor t = 3 h.The medium was replaced with 300 µL of fresh cell culture medium containing 10% FBS, and cells were imaged immediately. The µ-slides were maintained in a portable incubator (Pecon) at 37°C with 5% CO_2, which was coupled to the CLSM. The time lapse images were obtained using a Plan-Apochromat 10x/0.45 M2 objective. To minimize photo-bleaching and to avoid photo-cytoxicity, low laser power and proper time intervals (10 min) were used, and cells were imaged for 24 h. The auto-focus function of the microscope was performedby detecting the reflection of the bottom of the µ-slide.

To study the intranuclear distributions of polyplexes in two daughter cells right after mitosis, HeLa cells were seeded on µ-slide 8 well plates (1.0 cm^2 seeding area per well) at 2.4×10^4 cells per well in 300µL of culture medium one day prior to use. On the next day, cells were incubated with 300 µL of fresh serum free cell culture mediumcontaining$P_{1/1/0}$ or$P_{1/1/4}$ polyplexes at 1.4 µg/mL DNA^{Cy5} for t = 3 h. Cells were washed with PBS, and the nuclei were stained with 300µL of 1 µg/mL Hoechst 33342 for 10 min. Note that low concentration of Hoechst 33342 was usedin this case to avoid inhibition of cell division. Cells were observed using a Plan-Apochromat 63x/1.40 Oil DIC M27 objective, and cell divisionwas tracked (**Figure SI.III.6.1**). Right after mitosis (i.e., upon formation of two daughter cells), cells were scanned in the z-axis direction with an interval distance at 1µm. The intranuclear polyplexes were determined by orthogonal views (**Figure SI.III.6.2**). Here the data of $P_{1/1/0}$ are shown.

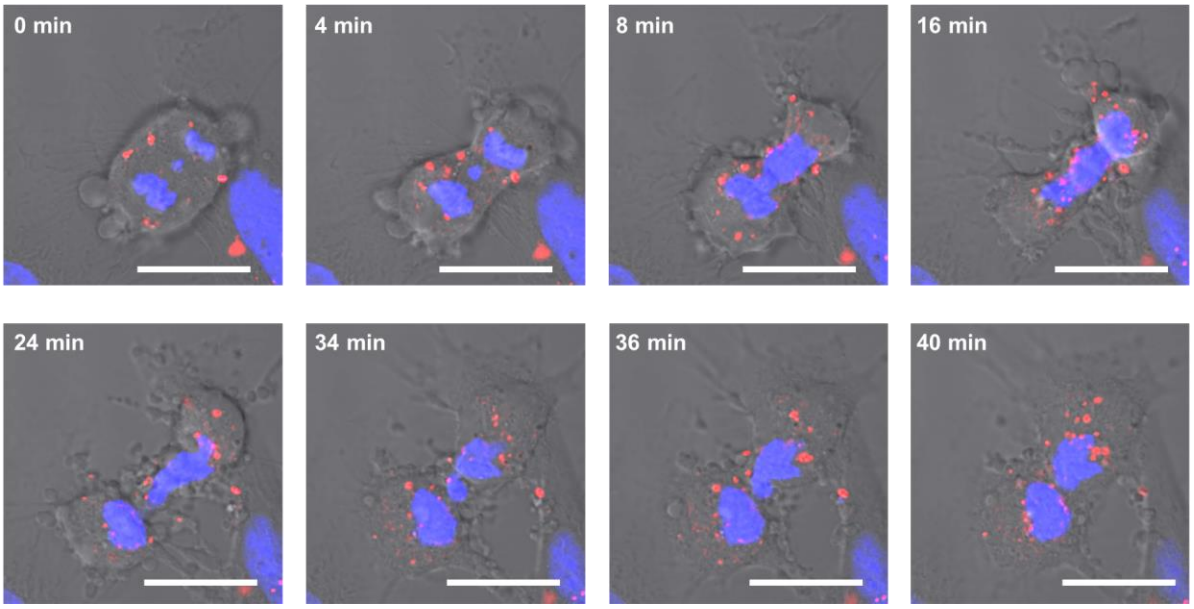

Figure SI.III.6.1. Time lapse images of cell division after transfection by $P_{1/1/0}$. Polyplexes containing DNA^{Cy5} are shown in red. Nuclei stained with Hoechst 33342 are shown in blue. The scale bars indicate 20 µm.

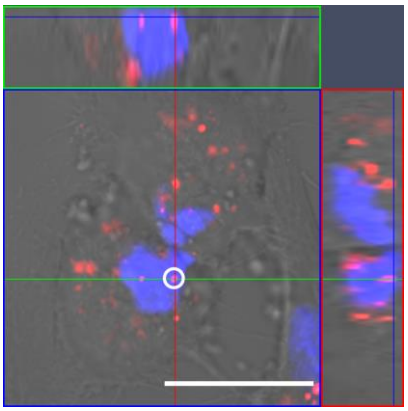

Figure SI.III.6.2. Orthogonal views of the polyplex distributions in two daughter cells. $P_{1/1/0}$containing DNA^{Cy5} is shown in red. Nuclei stained with Hoechst 33342 are shown in blue. The polyplex indicated by the circle is inside nucleus. The scale bar represents 20 µm.

IV) Image processing

IV.1) Calculation of percentages of cellsexpressing fluorescent proteins from images

Images for I_{eGFP} calculation were measured by a 10 × objective, and a pinhole value of 1 airy unit was applied for imaging. 1 airy unit = (0.61 × emission-wavelength × total Mag)/NA = 0.61 × 520 nm × 10/0.3 = 10.57 μm (https://www.med.unc.edu/microscopy/files/2018/06/clsm-tutorial-v2.pdf). Projection of cells with a thickness of 10.57 μm were acquired by the microscope, more or less the same height of a cell. Therefore, almost all the fluorescence in a whole cell was reflected in the images. The percentages of cells expressing only eGFP, RFP, or both proteins were analyzed from the images using the software Cellprofiler (v 3.1.8). As described in **III.3**, images acquired under 10× magnification were analyzed. As shown in **Figure SI.IV.1**, each raw image was split into 3 channels, including fluorescence from nuclei (blue), eGFP (green), and RFP (red). The nuclei were identified by the "IdentifyPrimaryObjectes" module using the "Otsu" as thresholding method. Their outlines are shown in green in **Figure SI.IV.1**. Too small nuclei (red outlines) and nuclei touching the borders (yellow outlines) were not counted. The identified nuclei were digitally shrunk to their center and then were expanded to circles with a diameter of 9 pixels. The eGFP and RFP fluorescence ($I_{eGFP}$and I_{RFP}) inside the circles was calculated, and the respective distributions were shown in the bar diagrams. In this way, the total integrated intensities of fluorescent proteins within the same area were compared. The white circles in **Figure SI.IV.1** indicate non-expressing cells, and yellow circles indicate expressing cells. In this way, we could do batch analysis and more than 10 images containing more than 5,000 cells in each condition were analyzed. To calculate the distributions of the eGFP fluorescence intensity (denoted as I_{eGFP}) in whole cellssuch as the ones shown in **Figure 2**b, similar methods were used, but nuclei were not shrunk to a circle. Instead, the eGFP signals were identified by the “IdentifySecondaryObjects” module, which was determined by “Propagation” method based on identified nuclei. 15 images containing more than 3,000 cells were analyzed. The data were saved in a file for further analysis in MATLAB 2013b.

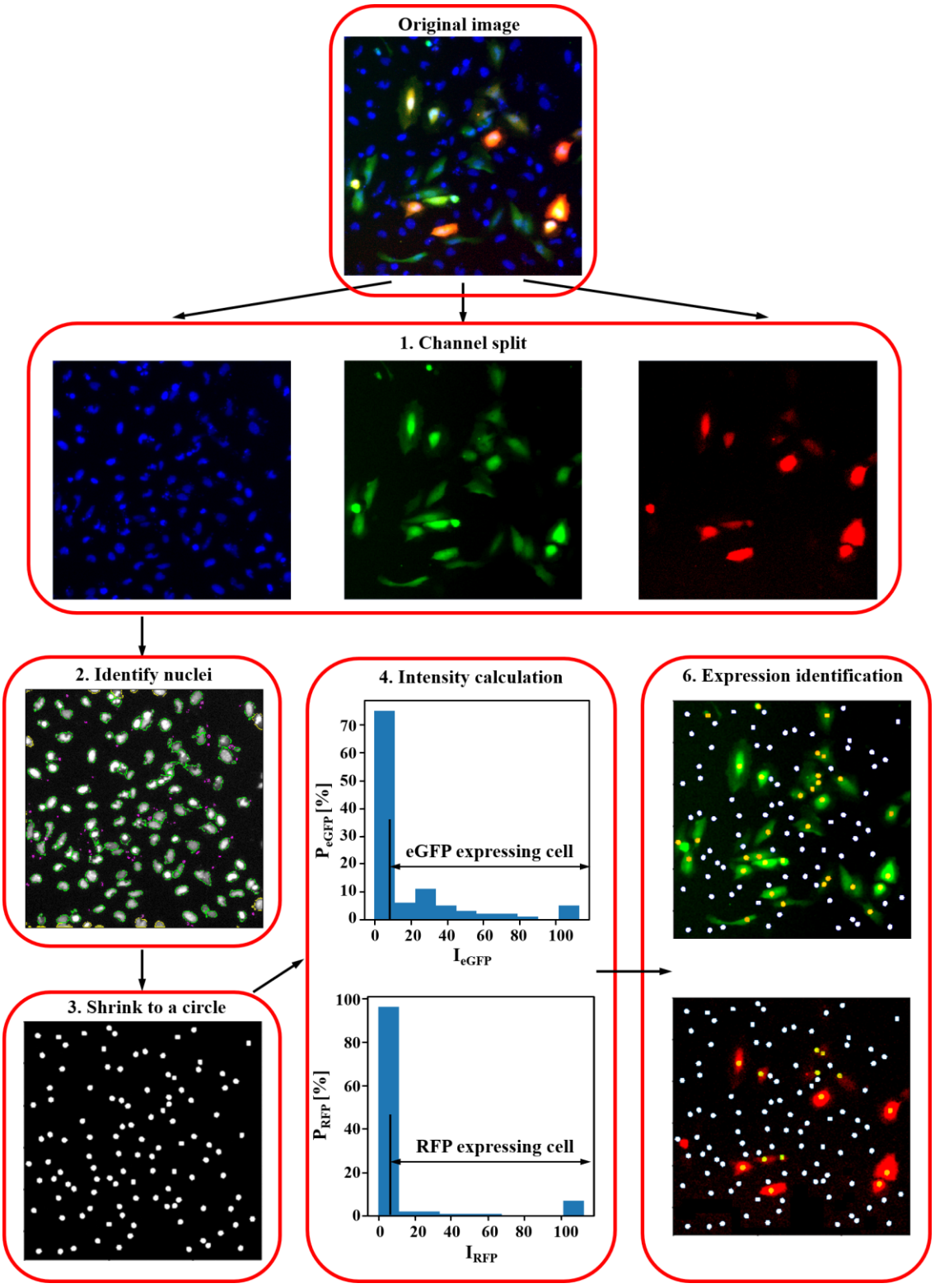

Figure SI.IV.1.1. Flowchart of finding out the thresholds for distinguishing non-expressing and expressing cells.

-

To calculate the percentages of cells only expressing eGFP, RFP or both proteins, the intensity thresholds of eGFPand RFP, i.e., $I_{eGFP,threshold}$ and $I_{RFP,threshold}$, were used. Their values were determined by the intensity distributions shown in step 4 in **Figure SI.IV.1**. Four situations would happen in our study: i) If $I_{eGFP}<I_{eGFP,threshold}$ and $I_{RFP}<I_{RFP,threshold}$, the cell was a non-expressing cell; ii) If $I_{eGFP}<I_{eGFP,threshold}$ and $I_{RFP}>I_{RFP,threshold}$, the cell only expressed RFP; iii) If $I_{eGFP}>I_{eGFP,threshold}$ and $I_{RFP}<I_{RFP,threshold}$, the cell only expressed GFP; iv) If $I_{eGFP}>I_{eGFP,threshold}$ and $I_{RFP}>I_{RFP,threshold}$, the cell expressed both. The cell number in each case was counted to get the percentages.

IV.2) Tracking cell migration, mitosis and eGFP expression

The identification of nuclei was a pre-requisite for cell tracking. However, the nuclei were not stained in this case, and thus we had to manually add “nuclei” to the images based on visual inspection by the software PhotoShop CS6.0. As shown in **Figure SI.IV.2**, a cell started to divide at 6 h 10 min, and the mitosis finished at 6 h 50 min. The “nuclei” in the center of cells were manually added accordingly as blue filled circles. Subsequently, the “nuclei” were identified in the software Cellprofiler (v 3.1.8) by the "Primary Objects" module. The eGFP signals were identified by the "Secondary Objects" module based on the identified nuclei regions, and their intensities were measured by the “MeasureObjectIntensity” module. As cells were always moving and some cells divided during observation, the trajectories of non-dividing cells and divided daughter cells from the same parents were recorded by the "TrackObjects" module using the "overlap" method. All the data were saved in a file for data plotting in MATLAB 2013. One representative data recording for $P_{1/1/0}$ mediated transfection is shown in **Movie SI.1**.

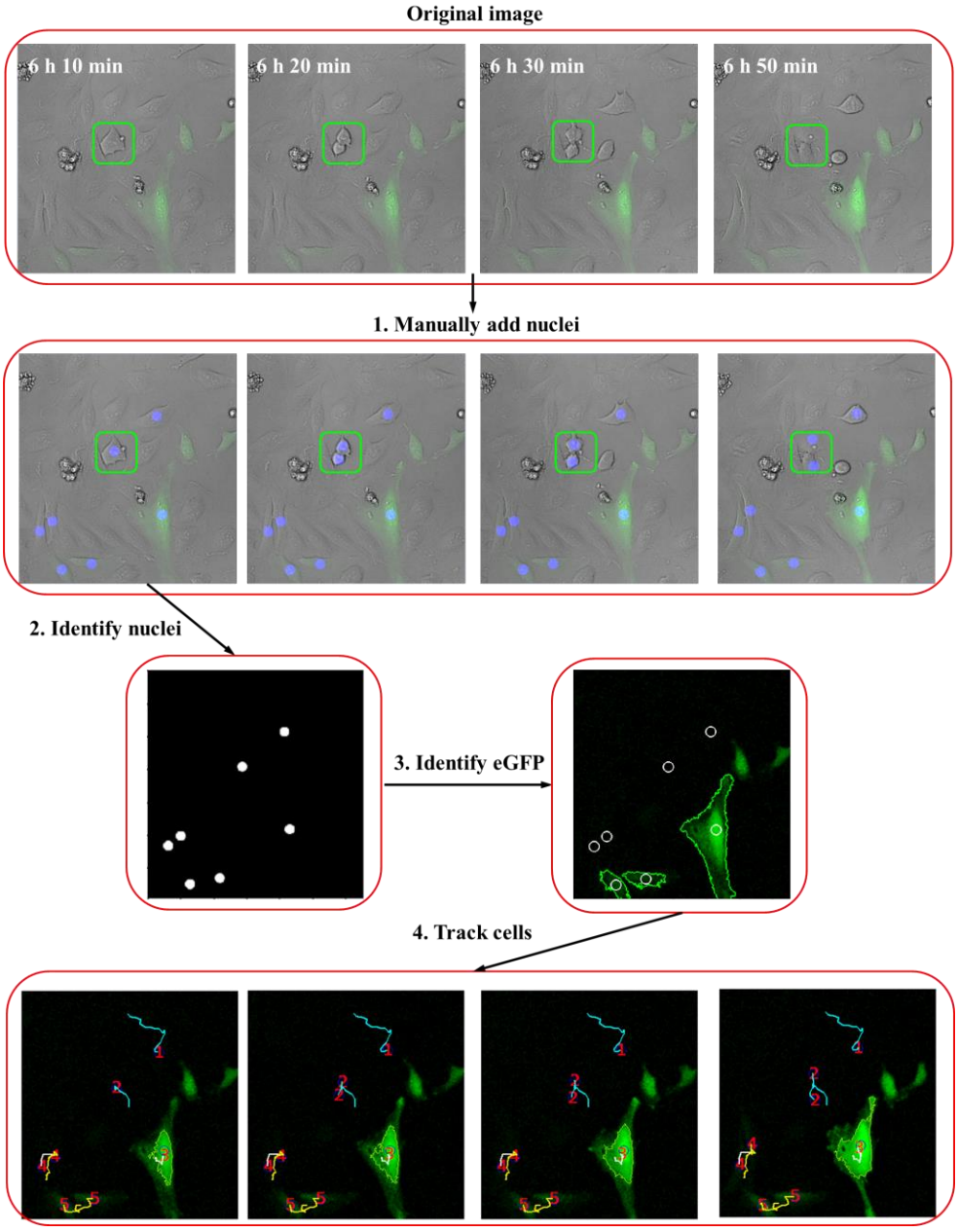

Figure SI.IV.2.1. Flowchart of tracking cells expressing eGFP.

IV.3) Colocalization as determined by Manders' coefficient calculation

All channels in the fluorescence images were smoothed to remove signal noise by a median filter, and the background (minimal pixel intensity) was subtracted using MATLAB 2013b. The fluorescent signals of DNA^{Cy5}, PEI^{RBITC}were identified, and their integrated fluorescence intensity was measured by Cellprofiler (v 3.1.8). Colocalization was quantified by calculating

their Manders' coefficients m_1 (DNA^{Cy5}) and m_2 (PEI^{RBITC}) according to the following equation[5]:

$$m_1 = \frac{\sum_i I(red)_{i,\,coloc}}{\sum_i I(red)_i}, m_2 = \frac{\sum_i I(green)_{i,\,coloc}}{\sum_i I(green)_i}$$

Manders' coefficients range from 0 to 1. One denotes complete co-localization and zero represents none. For m_1, $I(red)_{i,\,coloc}$ denotes the intensity of the red pixel iin case there is alsofluorescence in the green channel of the same pixel i, and $I(red)_i$ denotes the intensity of the red pixel i. The data processed by Cellprofiler were further analyzed by MATLAB 2013b to obtain Manders' coefficients.

An example is shown in **Figure SI.IV.3.1.** For discussion see also **Figure 6** of the main manuscript. Polyplexes were prepared from $DNA^{Cy5}$and also the PEI or the BSA was fluorescence labelled in the form of rhodamine Bisothiocyanate labelled PEI (PEI^{RBITC}) for $P_{1/1/0}$ and fluorescein labelled BSA (BSA^{FITC}) for $P_{1/1/4}$. HeLa cells were exposed to polyplexes in serum free culture medium for t = 3 h, followed by further culture in fresh medium (i.e., without polyplexes) containing 10% FBS for t' = 4 h, 10 h and 24 h, and recording by CLSM (**Figure SI.IV.3.1**a,b). The overlap degree was quantified by measuring the Mander's coefficients m_1 and m_2[6](**Figure SI.IV.3.1**c,d), which are indicators for the colocalization degree between pixels from two different fluorescence channels (i.e. red fluorescent DNA^{Cy5} and green fluorescent $PEI^{RBITC}$for $P_{1/1/0}$ and BSA^{FITC} for $P_{1/1/4}$)ranging from 0 to 1. Zero correlates to no overlap and one correlates to complete overlap. The coefficients m_1 of $P_{1/1/0}$ and$P_{1/1/0}$ were larger than 90% and 85%, respectively without significant difference at each time point, indicating most DNA molecules were still condensed inside the polyplexes.

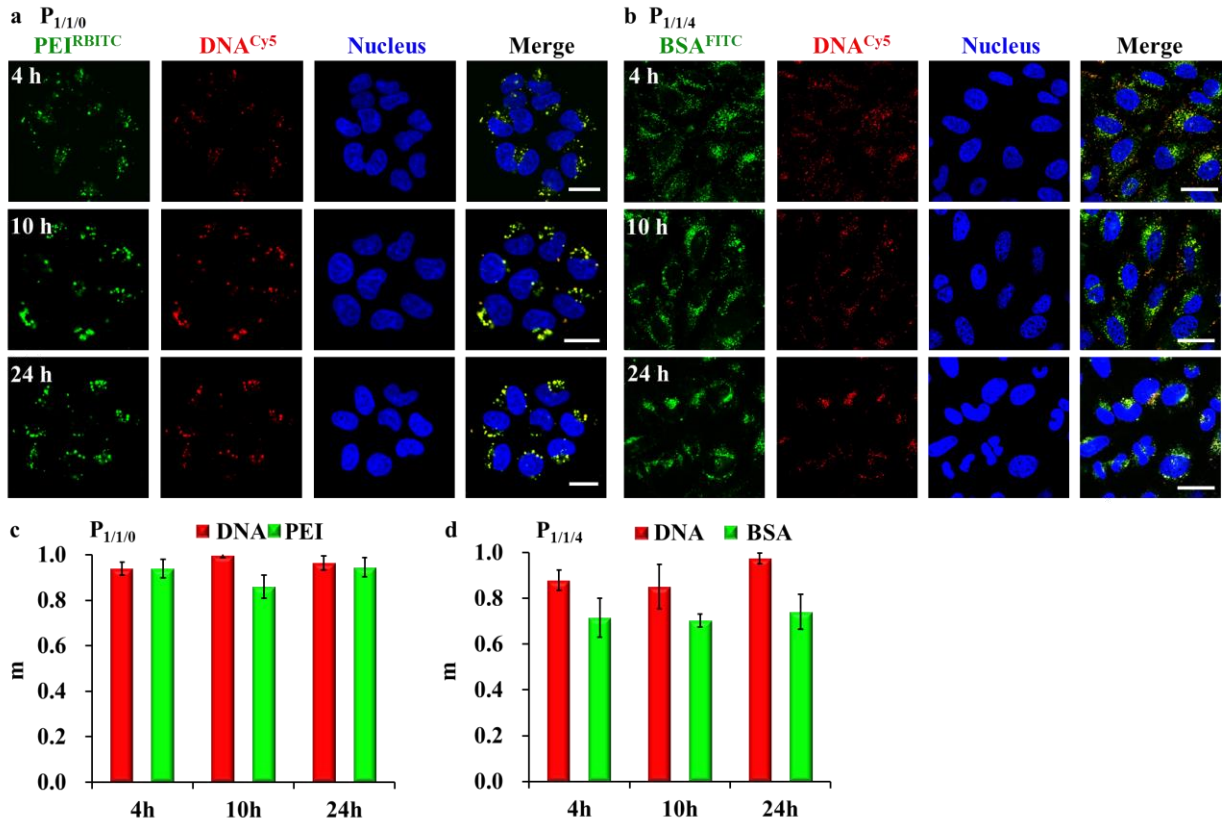

Figure SI.IV.3.1. Dissociation and nuclear entry of $P_{1/1/0}$ and $P_{1/1/4}$ polyplexes. a,b) HeLa cells were incubated with a) $P_{1/1/0}$ and b) $P_{1/1/4}$ polyplexes at 1.4 µg/mL DNA^{Cy5} in serum free culture medium for 3 h, followed by t' = 4 h, 10 h or 24 h of culture in fresh culture medium containing 10% FBS, but no polyplexes. In the fluorescence images DNA^{Cy5} is shown in the red, PEI^{RBITC} and BSA^{FITC} in the green fluorescence channel. Nucleus stained with Hoechst 33342 is seen in the blue fluorescence channel. The scale bars indicate 20 µm. c,d) Overlap degree of DNA^{Cy5} (red) and c) PEI^{RBITC} or d) BSA^{FITC} (green) calculated by pixel intensity. Red bars show Manders' coefficient m_1, the percentage of red fluorescent pixels overlapping with green fluorescent pixels. Green bars show Manders' coefficient m_2, the percentage of green fluorescent pixels overlapping with red fluorescent pixels. n≥50 cells in 4-5 images were analyzed for each group.

IV) References

[1] aD. Zhu, H. Yan, Z. Zhou, J. Tang, X. Liu, R. Hartmann, W. J. Parak, N. Feliu, Y. Shen, *Biomaterials Science* **2018**, *6*, 1800-1817; bX. Liu, J. Xiang, D. Zhu, L. Jiang, Z. Zhou, J. Tang, X. Liu, Y. Huang, Y. Shen, *Adv. Mater.* **2016**, *28*, 1743-1752; cD. Zhu, H. Yan, X. Liu, J. Xiang, Z. Zhou, J. Tang, X. Liu, Y. Shen, *Adv. Funct. Mater.* **2017**, *27*, 1606826.

[2] W. Zhang, X. Kang, B. Yuan, H. Wang, T. Zhang, M. Shi, Z. Zheng, Y. Zhang, C. Peng, X. Fan, *Theranostics* **2019**, *9*, 1580.

[3] C. Ganas, A. Weiß, M. Nazarenus, S. Rösler, T. Kissel, P. Rivera_Gil, W. J. Parak, *J. Control. Release* **2014**, *196*, 132-138.

[4] M. O. Durymanov, A. V. Yarutkin, Y. V. Khramtsov, A. A. Rosenkranz, A. S. Sobolev, *J. Control. Release* **2015**, *215*, 73-81.

[5] E. M. M. Manders, F. J. Verbeek, J. A. Aten, *Journal Of Microscopy-Oxford* **1993**, *169*, 375-382.

[6] aR. Hartmann, W. J. Parak, in *Systems Biology Application in Synthetic Biology* (Ed.: S. Singh), Springer India, Pune, **2016**, pp. 99-115; bC. Schweiger, R. Hartmann, F. Zhang, W. J. Parak, T. Kissel, P. Rivera Gil, *Journal of Nanobiotechnology* **2012**, *10*, 28.