Spatio-Temporal Cellular Imaging of Polymer-pDNA Nanocomplexes Affords In Situ Morphology and Trafficking Trends

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Supplementary Information

This Supplementary Information file includes:
Materials
Calculations for Polyplex Distributions
Dynamic Light Scattering Information
Figures S1 to S5
Tables S1 to S5
Movie S1Caption

Contents

Materials

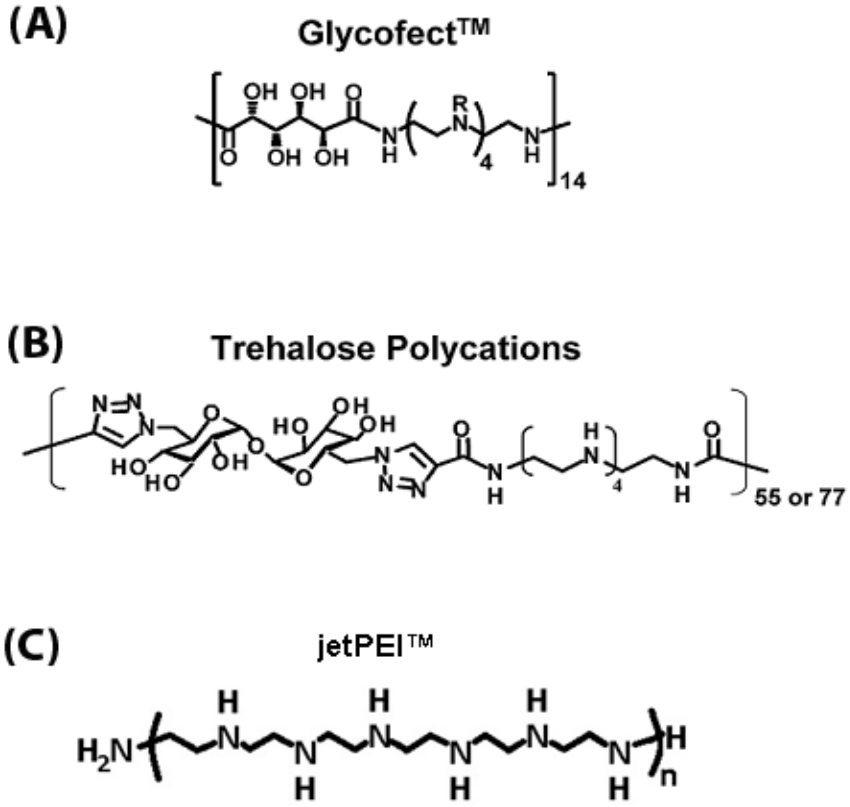


Figure S1: (A-C) Schematic structures of Glycofect™, Trehalose polycations ($Tr4_{55}$ and $Tr4_{77}$), and jetPEI™, respectively.

Confocal Slide Preparation

The cells on the coverslip were fixed with 100 μL/well of 4% (v/v) paraformaldehyde (ACROS Organics USA, NJ) for 15 minutes. After the cells were fixed, they were washed with PBS and permeabilized with 1 mL/well of 0.25% (v/v) Triton for 10 minutes. Next, the cells were washed three times with PBS (5 minutes each wash) and blocked with 1 mL/well of 1% (v/v) bovine albumin serum in PBS for 40 minutes. An equal concentration of rabbit polyclonal primary antibodies were used for both Rab 5 (raised against amino acid 1-215 representing full length Rab 5A of human origin; Mw = 25 kDa) and Rab 7 (raised against amino acids 158-207 mapping at C-terminus of Rab 7 of human origin; Mw = 22 kDa), in an effort to make the results directly comparable to each other. The dilutions (v/v) were 3 (Rab) : 500 (1% bovine serum albumin in PBS); and 1 mL/well was added to each well containing cells on glass coverslip to be treated. A goat anti-rabbit IgG secondary fluorescent antibody tagged Alexa Fluor® 555 was used to label the primary antibody. Secondary antibody dilutions (v/v) were 2.5 (Rab): 1000 (1% bovine serum albumin in PBS). For DAPI labeling, the coverslips were placed (cell side down) on 300 μL drops of DAPI solution on paraffin wax paper for 10 minutes. After final labeling with DAPI, the coverslips were mounted on glass slides (two per slide) on a 10 μL drop of ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA) and allowed to dry in the dark for 24 hours. After 24 hours, clear and colorless nail polish was used to seal the coverslips along their circumference and stored in -20 ^{o}C freezer until confocal imaging. Further, the clathrin primary antibody concentration used was 1 μg/mL and for caveolae the primary antibody dilution used was 1/500.[1] A similar procedure was used to that described above (and reported previously) for slide preparation.[1]

Calculations for Polyplex Distributions

The raw data was first binned (R) for the number of polyplexes (N) in all six cells with the following increments: 0.01 μm^3 for volume, 1 μm for distance from nucleus, and 0.5 μm for interpolyplex distance. The bin range 'r_{nf}' beyond 'r_{nn}' (please see footnotes below all of the tables) was not of interest because it represented data less than 10 to 20 % total data which was scattered over large number of bin ranges (up to 25 times 'r_{nn}').

The number of polyplexes in each of these bin ranges for six cells was added (n_{nn}). The total number of polyplexes from all of the individual bins was labeled as the 'total number of events' (n_t). To calculate probability (p_n), the number of polyplexes in each bin range (n_{nn}) was divided by the total number of polyplexes (n_t; or total events). In other words, the 'events, or n_n' divided by 'total number of events, or n_t' resulted in the probability value. This probability was the chance that the given number of polyplexes would exist at the given volume or distance at the given time point. The probability value (p_n), thus obtained, was plotted as the y-axis of the scatter plot, where the x-axis was the bin or range (r_n), (such as volume or distance) (

to S2).

For the volume distribution of the polyplexes, the Power Law model was fit to the data to obtain the equation and correlation coefficient (R^2-value). It should be noted that the fits were performed up to the bin range, which did not contain a zero number. The resulting equation was used to calculate the extrapolated probability values (P'). These values were plotted with the probability (p'_n) on the y-axis and time (T) on the x-axis for a varying bin range (r'_n) (

Note:

(i) "R" denotes "bin" or "range".
(ii) "P" denotes the probability.
(iii) "rn" denotes the respective "bin" or "range" shown in Table S1.
(iv) "P" is the probability calculated in Table S1.
(v) The inset plot of "R (x-axis: bin or range) vs P (y-axis: probability)". A trend-line of the Power-law fit to the data points was fitted. This resulted in an equation that was used to extrapolate various values. Tables S7 and S8 show that the power-law equation, thus generated, was a good fit in most cases.

to S5).

Table S1: Calculations for distributions

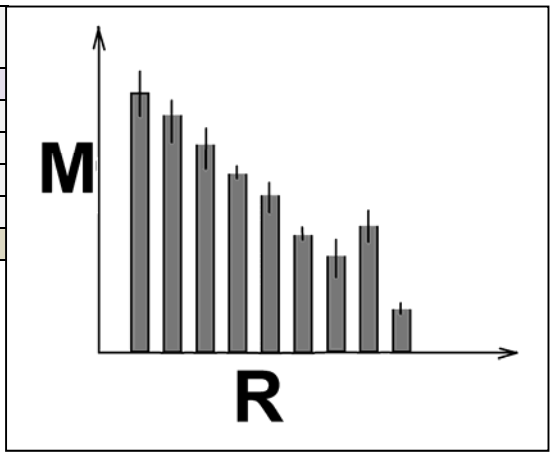
Polymer: A and time: T_n													**Distribution Calculations**		
Rab 5						**Rab 7**									
Cell # 1		**Cell # 2**		**Cell # 3**		**Cell # 4**		**Cell # 5**		**Cell # 6**				**Mean**	**Standard deviation**
R	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**		**R**	**M**	**SD**
r_{11}	n_{11}	r_{21}	n_{21}	r_{31}	n_{31}	r_{41}	n_{41}	r_{51}	n_{51}	r_{61}	n_{61}		r_1	$m_1= (n_{11}+ n_{21}+ n_{31}+ n_{41}+ n_{51}+ n_{61})/6$	s_1
r_{12}	n_{12}	r_{22}	n_{22}	r_{32}	n_{32}	r_{42}	n_{42}	r_{52}	n_{52}	r_{62}	n_{62}		r_2	$m_2= (n_{21}+ n_{22}+ n_{23}+ n_{24}+ n_{25}+ n_{26})/6$	s_2
:	:	:	:	:	:	:	:	:	:	:	:		:	:	:
r_{1n}	n_{1n}	r_{2n}	n_{2n}	r_{3n}	n_{3n}	r_{4n}	n_{4n}	r_{5n}	n_{5n}	r_{6n}	n_{6n}		r_n	$m_n= (n_{n1}+ n_{n2}+ n_{n3}+ n_{n4}+ n_{n5}+ n_{n6})/$	s_n
:	:	:	:	:	:	:	:	:	:	:	:				
r_{1f}	n_{1f}	r_{2f}	n_{2f}	r_{3f}	n_{3f}	r_{4f}	n_{4f}	r_{5f}	n_{5f}	r_{6f}	n_{6f}				

Note:
(i) "R" denotes "bin" or "range".
(ii) "N" denotes the number of polyplexes.
(iii) "r_{nn}" denotes the "bin" or "range" at respective "column = n" and "row = n"; For example: "r_{11}". denotes the "first" bin or range for "Cell # 1". And "r_{12}" denotes the "second" bin range for "Cell # 1". Thus "r_{1n}" denotes the "last" bin or range that was used in the calculations. "r_{nf}" denotes the last bin or range beyond "r_{nn}", which is not considered in the analysis because of very few events recorded.
(iv) For volume: "r_{nn}" varies from ">0.0 to 0.01", ">0.01 to 0.02"… … ">0.19 to 0.20".
(v) For distance from nucleus: "r_{nn}" varies from ">0 to 1", ">1 to 2"… … ">2 to 30".
(vi) For inter-polyplex distance: "r_{nn}" varies from ">0.0 to 0.5", ">0.5 to 1.0"… … ">9.5 to 10".
(vii) In the three values of range depicted in points "iv, v and vi" above, the last range (such as ">0.19 to 0.20" in case of volume distribution shown in point 'iv') for the power-law fit was the range listed as above (either ">0.19 to 0.20" for volume; or ">2 to 30" for distance from nucleus or ">9.5 to 10" for inter-polyplex distance). If a zero number was present in a range before this last range, then that particular range was considered last.
(viii) "T_n" denotes the data "R" and "N" at a time point of either 4, 8, 12 or 24 hours.
(ix) "r_n" denotes the same bin or range as listed in columns "R".

(x) "m_n" is the mean for all "six" cells at time point "T_n" for "one polymer". As shown, the mean is taken over the total number of polyplexes counted in that bin or range for all six cells.

Table S2: Plots for distribution data

Polymer: A and time: T_n		
R	**M**	**SD**
r_1	m_1	s_1
r_2	m_2	s_2
:	:	:
r_n	m_n	s_n



Note:

(i) "R" denotes the "bin" or "range".
(ii) "N" denotes the number of polyplexes.
(iii) "r_n" denotes the respective "bin" or "range" shown in Table S1.
(iv) "M" is the mean calculated in Table S1.
(v) "SD" is the standard deviation calculated in Table S1.
(vi) The inset plot of "R (x-axis: bin or range) vs M (y-axis: mean)" denotes the distributions. These are shown in Figure S2, S3, and S4.

Table S3: Calculations for probability data

Polymer: A and time: T_n													Probability Calculations		
Rab 5						Rab 7									
Cell # 1		Cell # 2		Cell # 3		Cell # 4		Cell # 5		Cell # 6				Total Number	Prob.
R	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**		**R**	**N_T**	**P**
r_{11}	n_{11}	r_{21}	n_{21}	r_{31}	n_{31}	r_{41}	n_{41}	r_{51}	n_{51}	r_{61}	n_{61}		r_1	$n_1 = (n_{11} + n_{21} + n_{31} + n_{41} + n_{51} + n_{61})$	$p_1 = n_1 / n_t$
r_{12}	n_{12}	r_{22}	n_{22}	r_{32}	n_{32}	r_{42}	n_{42}	r_{52}	n_{52}	r_{62}	n_{62}		r_2	$n_2 = (n_{21} + n_{22} + n_{23} + n_{24} + n_{25} + n_{26})$	$p_2 = n_2 / n_t$
:	:	:	:	:	:	:	:	:	:	:	:		:	:	:
r_{1n}	n_{1n}	r_{2n}	n_{2n}	r_{3n}	n_{3n}	r_{4n}	n_{4n}	r_{5n}	n_{5n}	r_{6n}	n_{6n}		r_n	$n_n = (n_{n1} + n_{n2} + n_{n3} + n_{n4} + n_{n5} + n_{n6})$	$p_n = n_n / n_t$
:	:	:	:	:	:	:	:	:	:	:	:	:		$n_t = (n_{n1} + n_{n2} + n_{n3} + n_{n4} + n_{n5} + n_{n6})$	
r_{1f}	n_{1f}	r_{2f}	n_{2f}	r_{3f}	n_{3f}	r_{4f}	n_{4f}	r_{5f}	n_{5f}	r_{6f}	n_{6f}				

Note:

(i) "R" denotes the "bin" or "range".
(ii) "N" denotes the number of polyplexes.

(iii) "r_{nn}" denotes the "bin" or "range" at respective "column = n" and "row = n"; For example: "r_{11}" denotes the "first" bin or range for "Cell # 1". And "r_{12}" denotes the "second" bin range for "Cell # 1". Thus, "r_{1n}" denotes the "last" bin or range that was used in calculations. "r_{nf}" denotes the last bin or range beyond "r_{nn}", which is not considered in the analysis because of very few events recorded.

(iv) For volume: "r_{nn}" varies from ">0.0 to 0.01", ">0.01 to 0.02"… … ">0.19 to 0.20".

(v) For distance from nucleus: "r_{nn}" varies from ">0 to 1", ">1 to 2"… … ">2 to 30".

(vi) For interpolyplex distance: "r_{nn}" varies from ">0.0 to 0.5", ">0.5 to 1.0"… … ">9.5 to 10".

(vii) In the three values of range depicted in points "iv, v and vi" above, the last range (such as ">0.19 to 0.20" in case of volume distribution shown in point 'iv') for the power-law fit was the range listed as above (either ">0.19 to 0.20" for volume; or ">2 to 30" for distance from nucleus or ">9.5 to 10" for inter-polyplex distance). If a zero number was present in a range before this last range, then that particular range was considered last

(viii) "T_n" denotes the data "R" and "N" at a time point of either 4, 8, 12 or 24 hours.

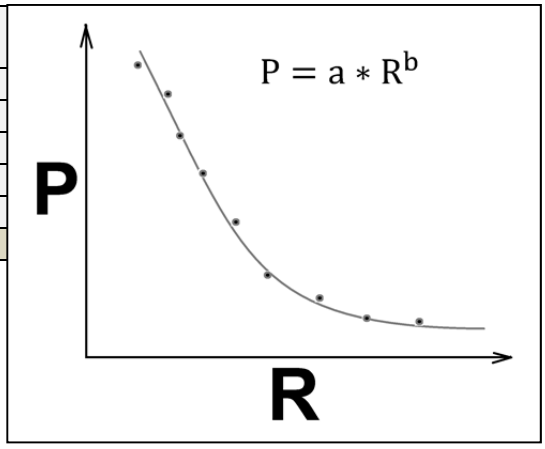
(ix) "r_n" denotes the same bin or range as listed in columns "R".

(x) "n_n" is the total number of polyplexes for all "six" cells at time point "T_n" for "one polymer".

(xi) "P" is the probability that is the ratio of "n_n" and "n_t", where "n_t" is the total number of polyplexes in all bins or ranges as shown above for one polymer at one time point.

Table S4: Power law fit to the data

Polymer: A and time: T_n	
R	**P**
r_1	p_1
r_2	p_2
:	:
r_n	p_n



Note:

(vi) "R" denotes "bin" or "range".

(vii) "P" denotes the probability.

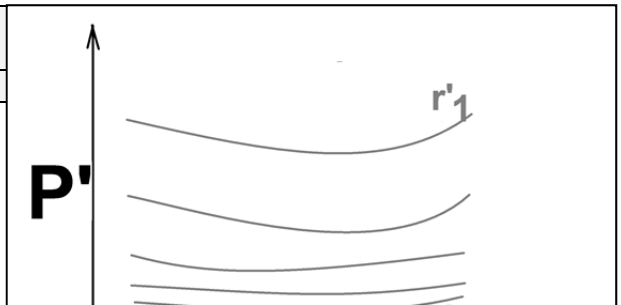
(viii) "r_n" denotes the respective "bin" or "range" shown in Table S1.

(ix) "P" is the probability calculated in Table S1.

(x) The inset plot of "R (x-axis: bin or range) vs P (y-axis: probability)". A trend-line of the Power-law fit to the data points was fitted. This resulted in an equation that was used to extrapolate various values. Tables S7 and S8 show that the power-law equation, thus generated, was a good fit in most cases.

Table S5: Plotting trends by extrapolating Power law fit equation

	Calculated probability (P') $P' = a * R'^{b}$			
R'	**4 hr**	**8 hr**	**12 hr**	**24 hr**



r'_1	$p'_{1\ @\ 4hr}$	$p'_{1\ @\ 8hr}$	$p'_{1\ @\ 12hr}$	$p'_{1\ @\ 24hr}$
r'_2	$p'_{2\ @\ 4hr}$	$p'_{2\ @\ 8hr}$	$p'_{2\ @\ 12hr}$	$p'_{2\ @\ 24hr}$
:	:	:	:	:
r'_n	$p'_{n\ @\ 4hr}$	$p'_{n\ @\ 8hr}$	$p'_{n\ @\ 12hr}$	$p'_{n\ @\ 24hr}$

Note:

(i) "R' " denotes extrapolated "bin" or "range".

(ii) "P' " denotes the extrapolated probability calculated from the power-law equation generated in Table S4.

(iii) "r'_n " denotes the respective "bin" or "range" shown in Table S1.

(iv) The inset plot of "T (x-axis: time point) vs. P' (y-axis: extrapolated probability)". The lines 'r'_n' show the trends generated. These are shown in Figure 2, 4, and 6.

Table S6: Nomenclature of bin ranges used in the manuscript.

Parameter	Units	Increment	Bin range	Notation	Example Bin range	Cited in manuscript
Volume	μm^3	0.01	> a to b	~b	>0.0 to 0.01	~0.01
Distance from nucleus	µm	1	> a to b	~b	>0 to 1	~1
Inter-polyplex distance	µm	0.5	> a to b	~b	>0.0 to 0.5	~0.5

As show in

, the number of polyplexes (n_n) in each of these bin ranges for six cells were added (n_{nn}). The total number of polyplexes from all of the individual bins were considered as the 'total number of events' (n_t). To calculate probability (p_n), the number of polyplexes in each bin range (n_{nn}) was divided by the total number of polyplexes (n_t; or total events). In other words, the 'events, n_n' divided by 'total number of events, n_t' resulted in the probability value. This probability was the chance that the given number of polyplexes would exist at the given volume

or distance at the given time point. The probability value (p_n), thus obtained, was plotted as the y-axis of the scatter plot, where the x-axis was the bin or range (r_n), (such as volume or distance),

.

Dynamic Light Scattering:

The autocorrelation function C(τ) was computed according to the equations below:

$$C(\tau) = Ae^{-2\Gamma t} + B \qquad \textbf{(Equation \# 1)}$$

where,

$$\Gamma = q^2 D \qquad \textbf{(Equation \# 2)}$$

$$q = \frac{4\pi n Sin\left(\frac{\theta}{2}\right)}{\lambda} \qquad \textbf{(Equation \# 3)}$$

$$D = \frac{k_B T}{3\pi\eta d} \qquad \textbf{(Equation \# 4)}$$

Nomenclature:

A, B: constants

t: time

Γ: decay or relaxation rate

τ: decay or relaxation time

q: scattering vector

D: diffusion coefficient

n: refractive index

θ: scattering angle

λ: laser wavelength

kB: Boltzmann's constant

T: temperature in Kelvin

η: liquid viscosity

d: particle diameter

Raw data was fit via an autocorrelation function (**Equation # 1**) to estimate parameters A, B, and Γ. The value for q was calculated from **Equation # 3**. These values for q and Γ were then used to calculate D from **Equation # 2**. Finally this D value was then used to calculate hydrodynamic diameter d from **Equation # 4**.

Supplementary Results

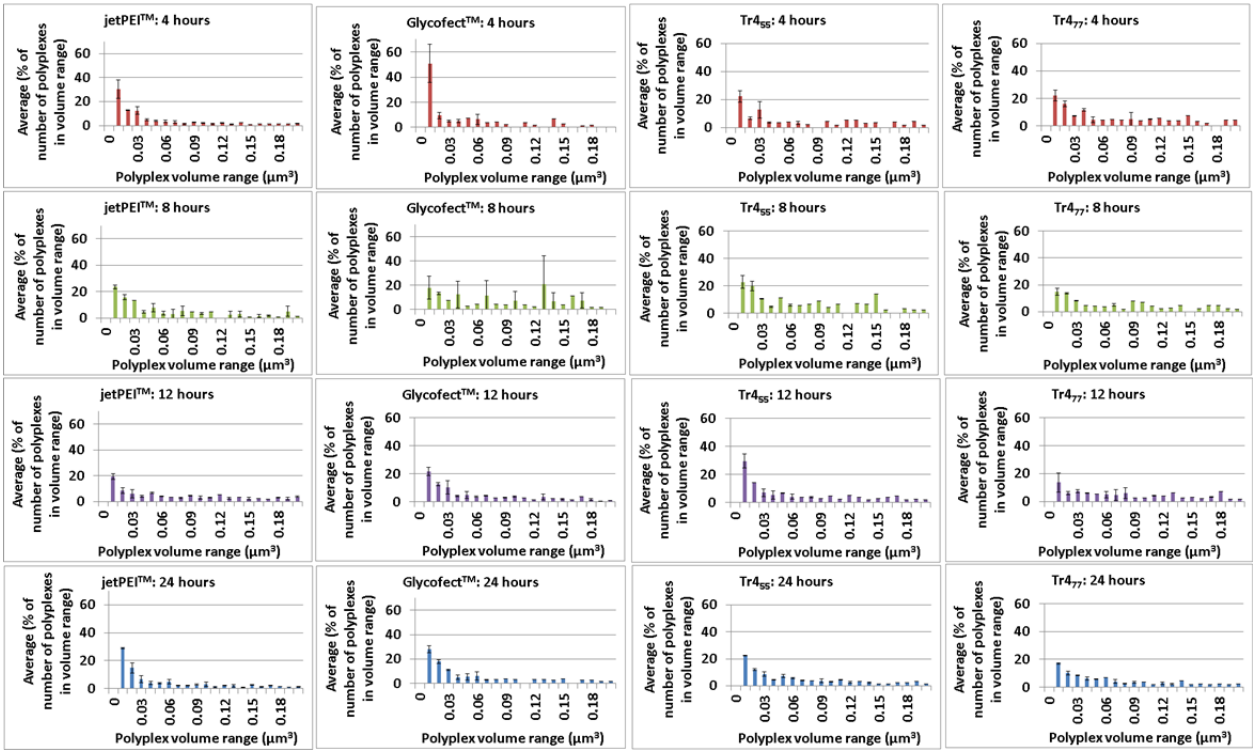


Figure S2: Volume distribution of polyplexes (in µm^3) in the intracellular environment. The columns indicate different polymers: jetPEI^{TM}, Glycofect^{TM}, Tr4$_{55}$, Tr4$_{77}$. As shown, the rows indicate the different post-transfection time points at which the cells were fixed. The Y-axis label indicates the average (of six cells) of the percentage (or fraction) of the number of polyplexes in a range. The percentage (or fraction) of the number of polyplexes in a range was calculated by dividing the number of polyplexes in a range by the total number of polyplexes in the entire cell (in all ranges). The error bars indicate the standard deviation.

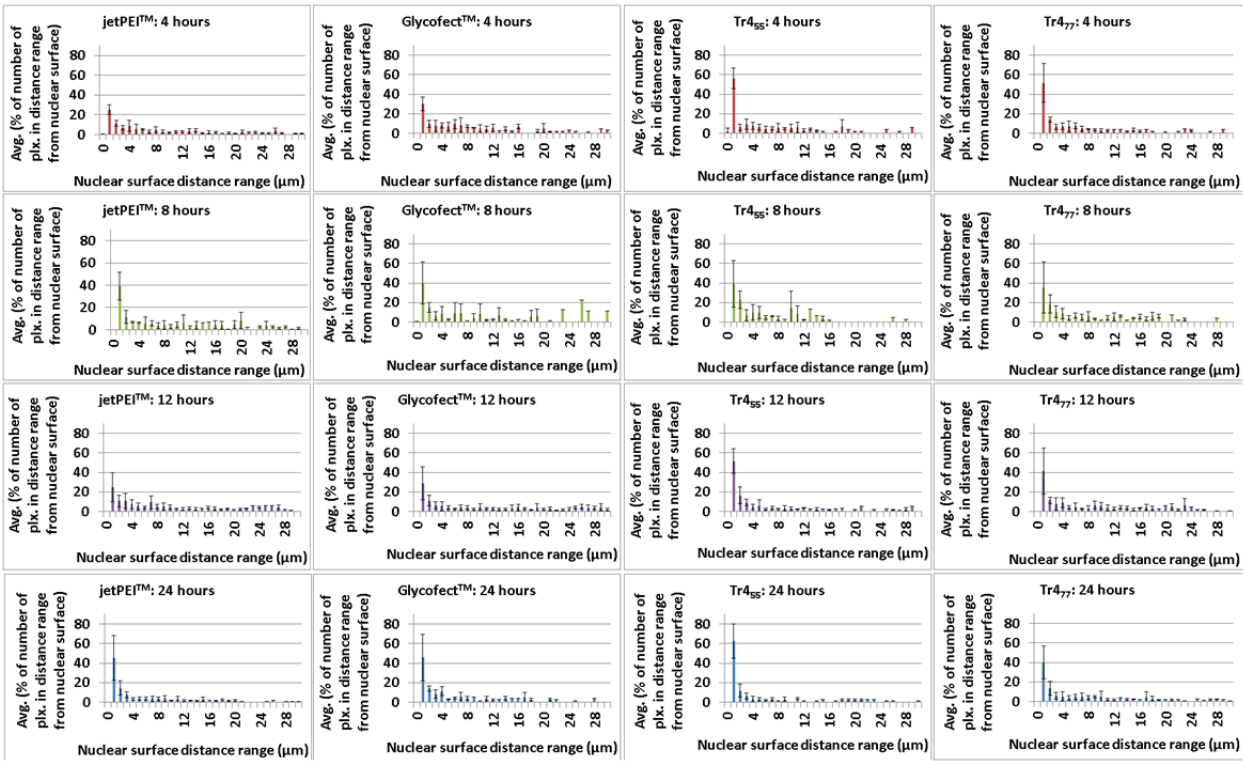


Figure S3: Polyplex distance from the surface of the nucleus (distribution of measurements in µm). The columns indicate data for polyplexes formed with each of the different polymers: jetPEI^{TM}, Glycofect^{TM}, Tr4$_{55}$, Tr4$_{77}$. As shown, the rows indicate different post-transfection time points at which the cells were fixed. The Y-axis label indicates the average percentage of the number of polyplexes in a range (for six cells). The percentage of the number of polyplexes in a range was calculated by dividing the number of polyplexes in that range by the total number of polyplexes in the entire cell (in all ranges). The error bars indicate the standard of deviation.

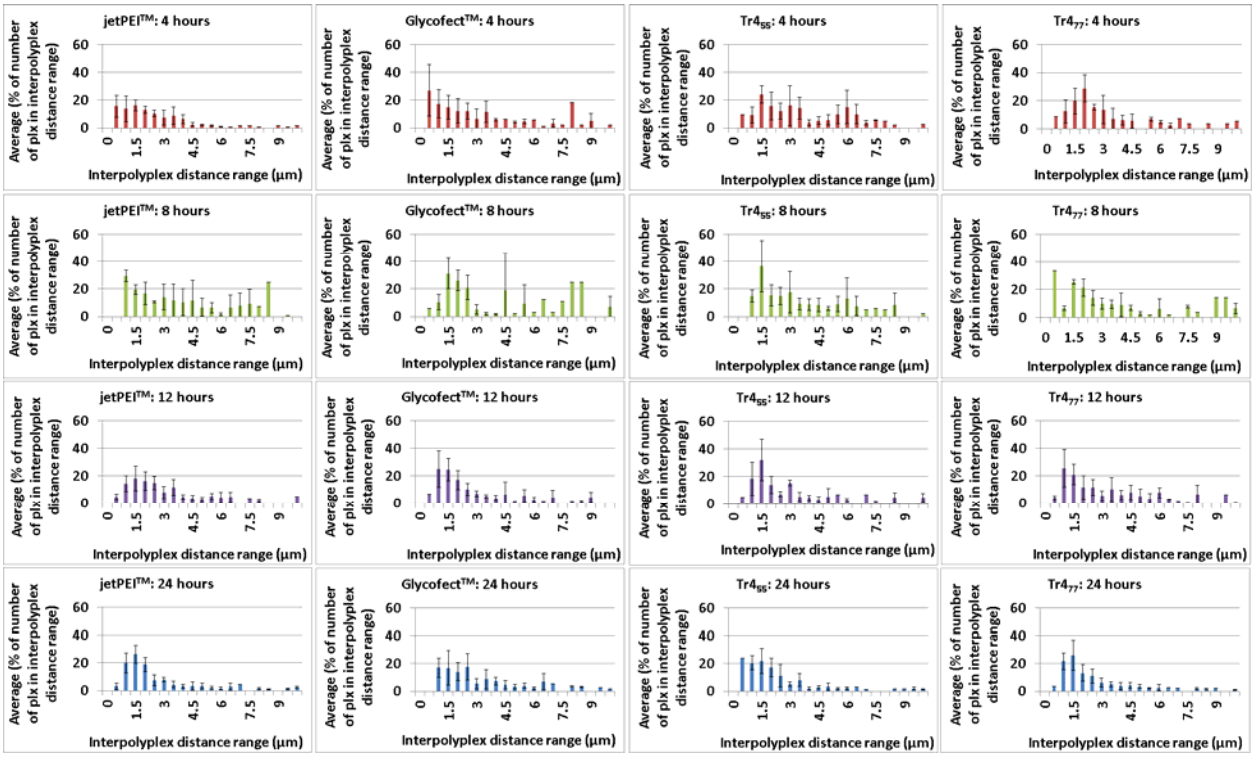


Figure S4: Distribution of measurements for the inter-polyplex distance (in µm). The columns indicate data for polyplexes formed with each of the different polymers: jetPEI^{TM}, Glycofect^{TM}, Tr4$_{55}$, Tr4$_{77}$. As shown, the rows indicate the different post-transfection time points at which the cells were fixed. The Y-axis label indicates the average percentage for the number of polyplexes in each range (for six cells). The percentage of the number of polyplexes in each range was calculated by dividing the number of polyplexes in each range by the total number of polyplexes in the entire cell (in all ranges). The error bars indicate the standard of deviation.

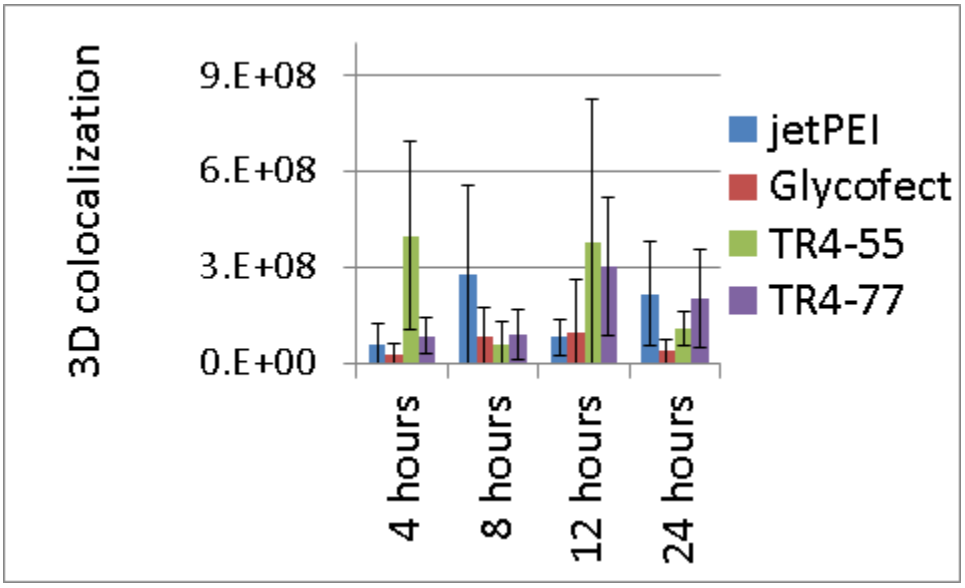


Figure S5: Sum of the colocalizing polyplex and nucleus voxel in 3D (arbitrary units) at 4, 8, 12, and 24 hour time points. Note: 3D colocalization refers to colocalization of voxels (similar to pixels in the case of 2D colocalization of confocal images). A voxel represents a volumetric pixel. None of the differences were statistically significant at alpha value of 0.05.

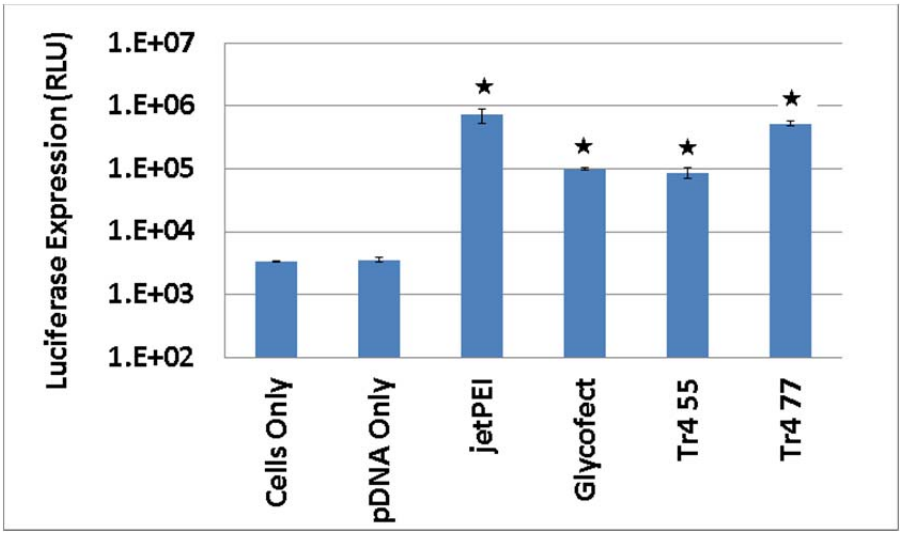


Figure S6: Luciferase gene expression in HeLa cells at 48 hours. The polyplexes consisting of respective polymers were allowed to transfect for 4 hours in reduced serum medium OptiMEM. Luminescence was measured as RLU (relative light units). Note: '*' indicates significant differences as compared to 'Cells Only' at alpha value of 0.05.

Table S7: Equations of fit, correlation (R^2), and goodness-of-fit values for intracellular polyplex volume.

Polymer Type		Post-transfection Timepoints			
		4 hour	**8 hour**	**12 hour**	**24 hour**
(A) Polyplex Volume Analysis					
jetPEI™	Power law fit (all)	$y = 0.0008x^{-1.309}$ $R^2 = 0.8952$	$y = 0.0015x^{-1.099}$ $R^2 = 0.8289$	$y = 0.0022x^{-0.946}$ $R^2 = 0.7965$	$y = 0.0014x^{-1.114}$ $R^2 = 0.8687$
	KS-Test (α=0.05)	$D_M = 0.0429 < D_C = 0.0837$	$D_M = 0.0297 < D_C = 0.1547$	$D_M = 0.0372 < D_C = 0.1447$	$D_M = 0.0519 < D_C = 0.0937$
Glycofect™	Power law fit (all)	$y = 0.0003x^{-1.583}$ $R^2 = 0.9285$	$y = 0.0016x^{-1.059}$ $R^2 = 0.6842$	$y = 0.0008x^{-1.329}$ $R^2 = 0.8563$	$y = 0.0005x^{-1.435}$ $R^2 = 0.9098$
	KS-Test (α=0.05)	$D_M = 0.0944 < D_C = 0.1351$	$D_M = 0.0506 < D_C = 0.1569$	$D_M = \underline{0.1078} > D_C = 0.0911$	$D_M = 0.1251 < D_C = 0.1467$
$Tr4_{55}$	Power law fit (all)	$y = 0.0004x^{-1.372}$ $R^2 = 0.891$	$y = 0.0007x^{-1.339}$ $R^2 = 0.74$	$y = 0.0006x^{-1.389}$ $R^2 = 0.8276$	$y = 0.001x^{-1.265}$ $R^2 = 0.8084$
	KS-Test (α=0.05)	$D_M = 0.0937 < D_C = 0.1982$	$D_M = 0.1086 < D_C = 0.1807$	$D_M = 0.1126 < D_C = 0.1246$	$D_M = \underline{0.1223} > D_C = 0.1038$
$Tr4_{77}$	Power law fit (all)	$y = 0.0007x^{-1.363}$ $R^2 = 0.855$	$y = 0.0014x^{-1.056}$ $R^2 = 0.7338$	$y = 0.0033x^{-0.798}$ $R^2 = 0.6545$	$y = 0.0017x^{-1.075}$ $R^2 = 0.8688$
	KS-Test (α=0.05)	$D_M = 0.1276 < D_C = 0.1418$	$D_M = 0.0994 < D_C = 0.1963$	$D_M = 0.0613 < D_C = 0.1453$	$D_M = 0.0882 < D_C = 0.1055$
(B) Nuclear Distance of Polyplex					
jetPEI™	Power law fit (all)	$y = 0.2658x^{-1.08}$ $R^2 = 0.7199$	$y = 0.2547x^{-1.152}$ $R^2 = 0.7667$	$y = 0.2039x^{-0.947}$ $R^2 = 0.7374$	$y = 0.4014x^{-1.388}$ $R^2 = 0.8397$
	KS-Test (α=0.05)	$D_M = 0.0611 < D_C = 0.0797$	$D_M = 0.1170 < D_C = 0.1355$	$D_M = 0.0265 < D_C = 0.1251$	$D_M = 0.0484 < D_C = 0.0829$
Glycofect™	Power law fit (all)	$y = 0.2969x^{-1.08}$ $R^2 = 0.7318$	$y = 0.2376x^{-1.182}$ $R^2 = 0.7451$	$y = 0.2161x^{-0.979}$ $R^2 = 0.8207$	$y = 0.3444x^{-1.377}$ $R^2 = 0.7543$
	KS-Test (α=0.05)	$D_M = 0.0835 < D_C = 0.1280$	$D_M = 0.1910 < D_C = 0.1488$	$D_M = 0.0814 < D_C = 0.0873$	$D_M = 0.0298 < D_C = 0.1364$
$Tr4_{55}$	Power law fit (all)	$y = 0.2777x^{-1.204}$ $R^2 = 0.8021$	$y = 0.3907x^{-1.426}$ $R^2 = 0.8589$	$y = 0.4701x^{-1.697}$ $R^2 = 0.9512$	$y = 0.5479x^{-2.056}$ $R^2 = 0.8901$
	KS-Test (α=0.05)	$D_M = 0.1734 < D_C = 0.1478$	$D_M = 0.0412 < D_C = 0.1643$	$D_M = 0.0343 < D_C = 0.1179$	$D_M = 0.0179 < D_C = 0.1092$
$Tr4_{77}$	Power law fit (all)	$y = 0.3732x^{-1.381}$ $R^2 = 0.8744$	$y = 0.2604x^{-1.093}$ $R^2 = 0.7455$	$y = 0.2152x^{-0.993}$ $R^2 = 0.6828$	$y = 0.3775x^{-1.353}$ $R^2 = 0.8691$
	KS-Test (α=0.05)	$D_M = 0.0697 < D_C = 0.1383$	$D_M = 0.0613 < D_C = 0.1562$	$D_M = 0.0846 < D_C = 0.1198$	$D_M = 0.0338 < D_C = 0.0956$
(C) Inter-polyplex Distance Analysis					

jetPEI™	Power law fit (all)	$y = 1.4335x^{-3.033}$ $R^2 = 0.8563$	$y = 0.6003x^{-2.013}$ $R^2 = 0.9118$	$y = 0.5989x^{-1.911}$ $R^2 = 0.8828$	$y = 0.8394x^{-2.415}$ $R^2 = 0.9729$
	KS-Test (α=0.05)	$D_M = \underline{0.1863} > D_C = 0.0989$	$D_M = 0.07705 < D_C = 0.1403$	$D_M = 0.0916 < D_C = 0.1308$	$D_M = 0.0553 < D_C = 0.0932$
Glycofect™	Power law fit (all)	$y = 0.3755x^{-1.961}$ $R^2 = 0.8498$	$y = 0.7072x^{-2.356}$ $R^2 = 0.8178$	$y = 1.181x^{-2.86}$ $R^2 = 0.8671$	$y = 0.6261x^{-2.087}$ $R^2 = 0.8819$
	KS-Test (α=0.05)	$D_M = 0.0638 < D_C = 0.1666$	$D_M = 0.0897 < D_C = 0.1517$	$D_M = 0.0549 < D_C = 0.1280$	$D_M = 0.1119 < D_C = 0.1466$
$Tr4_{55}$	Power law fit (all)	$y = 0.6541x^{-2.004}$ $R^2 = 0.8000$	$y = 0.5811x^{-1.888}$ $R^2 = 0.7588$	$y = 0.89x^{-2.492}$ $R^2 = 0.9341$	$y = 0.9009x^{-2.612}$ $R^2 = 0.7889$
	KS-Test (α=0.05)	$D_M = 0.1158 < D_C = 0.1436$	$D_M = 0.1369 < D_C = 0.1666$	$D_M = 0.0125 < D_C = 0.1289$	$D_M = \underline{0.1199} > D_C = 0.1152$
$Tr4_{77}$	Power law fit (all)	$y = 0.5278x^{-1.703}$ $R^2 = 0.8328$	$y = 0.9693x^{-2.411}$ $R^2 = 0.8231$	$y = 0.5158x^{-2.079}$ $R^2 = 0.8700$	$y = 0.9039x^{-2.464}$ $R^2 = 0.8695$
	KS-Test (α=0.05)	$D_M = 0.0866 < D_C = 0.1506$	$D_M = 0.1497 < D_C = 0.1587$	$D_M = 0.0946 < D_C = 0.1434$	$D_M = 0.0979 < D_C = 0.1065$

Table S8: P-values for the normal distribution of data points in each individual by Shapiro-Wilk W Test.

Polymer Type	Bin or Range	Post-transfection Timepoints			
		4 hour	8 hour	12 hour	24 hour
(A) Number of polyplexes in a volume range (n = 6 per range)					
jetPEI™	>0.00 to 0.01	p = 0.1644 > α = 0.05	p = 0.9366 > α = 0.05	p = 0.1357 > α = 0.05	p = 0.9940 > α = 0.05
	>0.01 to 0.02	p = 0.5263 > α = 0.05	p = 0.6931 > α = 0.05	p = 0.9168 > α = 0.05	p = 0.2874 > α = 0.05
	>0.02 to 0.03	p = 0.7072 > α = 0.05	p = 0.0196 < α = 0.05	p = 0.9987 > α = 0.05	p = 0.0055 < α = 0.05
	>0.03 to 0.04	p = 0.6950 > α = 0.05	p = 0.0726 > α = 0.05	p = 0.4922 > α = 0.05	p = 0.6379 > α = 0.05
	>0.04 to 0.05	p = 0.9852 > α = 0.05	p = 0.1221 > α = 0.05	p = 0.3768 > α = 0.05	p = 0.0452 < α = 0.05
	> 0.06 to 0.20	p = 0.9703 > α = 0.05	p = 0.6889 > α = 0.05	p = 0.2601 > α = 0.05	p = 0.8399 > α = 0.05
Glycofect™	>0.00 to 0.01	p = 0.6952 > α = 0.05	p = 0.4944 > α = 0.05	p = 0.1238 > α = 0.05	p = 0.4656 > α = 0.05
	>0.01 to 0.02	p = 0.4321 > α = 0.05	p = 0.0690 > α = 0.05	p = 0.7409 > α = 0.05	p = 0.8189 > α = 0.05
	>0.02 to 0.03	p = 0.9926 > α = 0.05	p = 0.0382 < α = 0.05	p = 0.0820 > α = 0.05	p = 0.9612 > α = 0.05
	>0.03 to 0.04	p = 0.2008 > α = 0.05	p = 0.0410 < α = 0.05	p = 0.1485 > α = 0.05	p = 0.8762 > α = 0.05
	>0.04 to 0.05	p = 0.0771 > α = 0.05	p = 0.0410 < α = 0.05	p = 0.0007 < α = 0.05	p = 0.6321 > α = 0.05
	> 0.06 to 0.20	p = 0.4086 > α = 0.05	p = 0.0376 > α = 0.05	p = 0.7059 > α = 0.05	p = 0.9649 > α = 0.05
$Tr4_{55}$	>0.00 to 0.01	p = 0.8928 > α = 0.05	p = 0.8472 > α = 0.05	p = 0.5070 > α = 0.05	p = 0.5558 > α = 0.05
	>0.01 to 0.02	p = 0.5407 > α = 0.05	p = 0.5551 > α = 0.05	p = 0.5879 > α = 0.05	p = 0.5834 > α = 0.05
	>0.02 to 0.03	p = 0.2749 > α = 0.05	p = 0.5530 > α = 0.05	p = 0.0483 < α = 0.05	p = 0.7743 > α = 0.05
	>0.03 to 0.04	p = 0.9895 > α = 0.05	p = 0.0195 < α = 0.05	p = 0.0097 < α = 0.05	p = 0.1452 > α = 0.05
	>0.04 to 0.05	p = 0.1110 > α = 0.05	p = 0.0858 > α = 0.05	p = 0.8465 > α = 0.05	p = 0.8223 > α = 0.05
	> 0.06 to 0.20	p = 0.9498 > α = 0.05	p = 0.7450 > α = 0.05	p = 0.6441 > α = 0.05	p = 0.0470 > α = 0.05
$Tr4_{77}$	>0.00 to 0.01	p = 0.7954 > α = 0.05	p = 0.9530 > α = 0.05	p = 0.5745 > α = 0.05	p = 0.4075 > α = 0.05
	>0.01 to 0.02	p = 0.8212 > α = 0.05	p = 0.4210 > α = 0.05	p = 0.9836 > α = 0.05	p = 0.7143 > α = 0.05
	>0.02 to 0.03	p = 0.2907 > α = 0.05	p = 0.7516 > α = 0.05	p = 0.8447 > α = 0.05	p = 0.9466 > α = 0.05
	>0.03 to 0.04	p = 0.6588 > α = 0.05	p = 0.0488 < α = 0.05	p = 0.4271 > α = 0.05	p = 0.6871 > α = 0.05
	>0.04 to 0.05	p = 0.9834 > α = 0.05	p = 0.1250 > α = 0.05	p = 0.0065 < α = 0.05	p = 0.7178 > α = 0.05
	> 0.06 to 0.20	p = 0.5269 > α = 0.05	p = 0.4334 > α = 0.05	p = 0.8418 > α = 0.05	p = 0.8066 > α = 0.05
(B) Distance range of polyplex from surface of the nucleus (n = 6 per range)					
jetPEI™	>0 to 1	p = 0.7010 > α = 0.05	p = 0.7534 > α = 0.05	p = 0.2442 > α = 0.05	p = 0.8312 > α = 0.05
	>1 to 2	p = 0.6553 > α = 0.05	p = 0.2548 > α = 0.05	p = 0.9583 > α = 0.05	p = 0.0197 < α = 0.05
	>2 to 3	p = 0.2436 > α = 0.05	p = 0.0213 < α = 0.05	p = 0.6058 > α = 0.05	p = 0.0568 > α = 0.05
	>3 to 4	p = 0.7336 > α = 0.05	p = 0.0136 < α = 0.05	p = 0.6096 > α = 0.05	p = 0.6566 > α = 0.05
	>4 to 5	p = 0.0953 > α = 0.05	p = 0.0079 < α = 0.05	p = 0.5963 > α = 0.05	p = 0.6586 > α = 0.05
Glycofect™	>0 to 1	p = 0.3696 > α = 0.05	p = 0.6446 > α = 0.05	p = 0.0735 > α = 0.05	p = 0.0840 > α = 0.05
	>1 to 2	p = 0.7111 > α = 0.05	p = 0.2737 > α = 0.05	p = 0.7623 > α = 0.05	p = 0.4755 > α = 0.05
	>2 to 3	p = 0.1649 > α = 0.05	p = 0.2569 > α = 0.05	p = 0.3432 > α = 0.05	p = 0.8413 > α = 0.05
	>3 to 4	p = 0.9102 > α = 0.05	p = 0.0199 < α = 0.05	p = 0.0190 < α = 0.05	p = 0.2680 > α = 0.05
	>4 to 5	p = 0.3394 > α = 0.05	p = 0.0054 < α = 0.05	p = 0.3537 > α = 0.05	p = 0.0023 < α = 0.05
$Tr4_{55}$	>0 to 1	p = 0.1131 > α = 0.05	p = 0.9956 > α = 0.05	p = 0.6551 > α = 0.05	p = 0.9243 > α = 0.05
	>1 to 2	p = 0.7946 > α = 0.05	p = 0.4643 > α = 0.05	p = 0.5510 > α = 0.05	p = 0.7754 > α = 0.05
	>2 to 3	p = 0.7183 > α = 0.05	p = 0.0919 > α = 0.05	p = 0.1780 > α = 0.05	p = 0.0086 < α = 0.05
	>3 to 4	p = 0.8433 > α = 0.05	p = 0.2037 > α = 0.05	p = 0.1608 > α = 0.05	p = 0.1248 > α = 0.05
	>4 to 5	p = 0.1207 > α = 0.05	p = 0.0527 > α = 0.05	p = 0.5747 > α = 0.05	p = 0.4971 > α = 0.05
$Tr4_{77}$	>0 to 1	p = 0.6746 > α = 0.05	p = 0.2068 > α = 0.05	p = 0.0649 > α = 0.05	p = 0.5152 > α = 0.05
	>1 to 2	p = 0.1447 > α = 0.05	p = 0.2479 > α = 0.05	p = 0.3258 > α = 0.05	p = 0.7820 > α = 0.05
	>2 to 3	p = 0.6413 > α = 0.05	p = 0.7269 > α = 0.05	p = 0.0927 > α = 0.05	p = 0.0853 > α = 0.05
	>3 to 4	p = 0.4823 > α = 0.05	p = 0.2904 > α = 0.05	p = 0.9225 > α = 0.05	p = 0.1083 > α = 0.05
	>4 to 5	p = 0.0274 < α = 0.05	p = 0.1006 > α = 0.05	p = 0.1268 > α = 0.05	p = 0.1314 > α = 0.05

(C) Inter-polyplex distance range (n = 6 per range)					
jetPEI™	>0.0 to 0.5	p = 0.2696 > α = 0.05	p = ---	p = 0.0067 < α = 0.05	p = 0.0173 < α = 0.05
	>0.5 to 1.0	p = 0.0012 < α = 0.05	p = 0.0038 < α = 0.05	p = 0.4781 > α = 0.05	p = 0.3955 > α = 0.05
	>1.0 to 1.5	p = 0.2787 > α = 0.05	p = 0.1007 > α = 0.05	p = 0.9918 > α = 0.05	p = 0.3317 > α = 0.05
	>1.5 to 2.0	p = 0.4719 > α = 0.05	p = 0.9504 > α = 0.05	p = 0.8098 > α = 0.05	p = 0.0744 > α = 0.05
	>2.0 to 2.5	p = 0.2883 > α = 0.05	p = 0.0099 < α = 0.05	p = 0.4176 > α = 0.05	p = 0.7804 > α = 0.05
	>2.5 to 3.0	p = 0.1560 > α = 0.05	p = 0.6272 > α = 0.05	p = 0.5033 > α = 0.05	p = 0.9009 > α = 0.05
Glycofect™	>0.0 to 0.5	p = 0.0182 < α = 0.05	p = 0.0001 < α = 0.05	p = 0.0001 < α = 0.05	p = ---
	>0.5 to 1.0	p = 0.3640 > α = 0.05	p = 0.0304 < α = 0.05	p = 0.8251 > α = 0.05	p = 0.6809 > α = 0.05
	>1.0 to 1.5	p = 0.6134 > α = 0.05	p = 0.1145 > α = 0.05	p = 0.7304 > α = 0.05	p = 0.5575 > α = 0.05
	>1.5 to 2.0	p = 0.6531 > α = 0.05	p = 0.2425 > α = 0.05	p = 0.6302 > α = 0.05	p = 0.6611 > α = 0.05
	>2.0 to 2.5	p = 0.9932 > α = 0.05	p = 0.5836 > α = 0.05	p = 0.8466 > α = 0.05	p = 0.9795 > α = 0.05
	>2.5 to 3.0	p = 0.0496 < α = 0.05	p = 0.0425 > α = 0.05	p = 0.5733 > α = 0.05	p = 0.5006 > α = 0.05
$Tr4_{55}$	>0.0 to 0.5	p = 0.0001 < α = 0.05	p = ---	p = 0.0001 < α = 0.05	p = 0.0001 < α = 0.05
	>0.5 to 1.0	p = 0.7041 > α = 0.05	p = 0.0067 < α = 0.05	p = 0.1251 > α = 0.05	p = 0.1365 > α = 0.05
	>1.0 to 1.5	p = 0.2765 > α = 0.05	p = 0.1133 > α = 0.05	p = 0.4262 > α = 0.05	p = 0.1804 > α = 0.05
	>1.5 to 2.0	p = 0.1185 > α = 0.05	p = 0.1212 > α = 0.05	p = 0.4226 > α = 0.05	p = 0.1313 > α = 0.05
	>2.0 to 2.5	p = 0.9727 > α = 0.05	p = 0.9059 > α = 0.05	p = 0.8822 > α = 0.05	p = 0.8302 > α = 0.05
	>2.5 to 3.0	p = 0.1804 > α = 0.05	p = 0.0538 > α = 0.05	p = 0.0284 < α = 0.05	p = 0.6082 > α = 0.05
$Tr4_{77}$	>0.0 to 0.5	p = 0.0001 < α = 0.05	p = 0.0001 < α = 0.05	p = 0.0069 < α = 0.05	p = 0.0001 < α = 0.05
	>0.5 to 1.0	p = 0.7076 > α = 0.05	p = 0.0405 < α = 0.05	p = 0.9136 > α = 0.05	p = 0.1493 > α = 0.05
	>1.0 to 1.5	p = 0.9884 > α = 0.05	p = 0.0093 < α = 0.05	p = 0.8603 > α = 0.05	p = 0.8705 > α = 0.05
	>1.5 to 2.0	p = 0.3748 > α = 0.05	p = 0.4501 > α = 0.05	p = 0.5549 > α = 0.05	p = 0.0195 < α = 0.05
	>2.0 to 2.5	p = 0.0181 > α = 0.05	p = 0.4321 > α = 0.05	p = 0.2339 > α = 0.05	p = 0.1432 > α = 0.05
	>2.5 to 3.0	p = 0.3435 > α = 0.05	p = 0.4916 > α = 0.05	p = 0.4450 > α = 0.05	p = 0.6621 > α = 0.05
(D) Colocalization of pDNA complexes with nucleus (n = 6)					
jetPEI™		p = 0.0300 < α = 0.05	p = 0.4273 > α = 0.05	p = 0.2728 > α = 0.05	p = 0.1659 > α = 0.05
Glycofect™		p = 0.1926 > α = 0.05	p = 0.1762 > α = 0.05	p = 0.0018 < α = 0.05	p = 0.0342 < α = 0.05
$Tr4_{55}$		p = 0.3952 > α = 0.05	p = 0.0812 > α = 0.05	p = 0.0346 < α = 0.05	p = 0.7054 > α = 0.05
$Tr4_{77}$		p = 0.5834 > α = 0.05	p = 0.0959 > α = 0.05	p = 0.8517 > α = 0.05	p = 0.7524 > α = 0.05
(E) Colocalization of pDNA complexes with Rab protein (n = 3)					
jetPEI™	Rab 5	p = 0.3713 > α = 0.05	p = 0.0216 < α = 0.05	p = 0.3156 > α = 0.05	p = 0.7676 > α = 0.05
	Rab 7	p = 0.2001 > α = 0.05	p = 0.4266 > α = 0.05	p = 0.4142 > α = 0.05	p = 0.0838 > α = 0.05
Glycofect™	Rab 5	p = 0.2582 > α = 0.05	p = 0.3531 > α = 0.05	p = 0.3318 > α = 0.05	p = 0.7281 > α = 0.05
	Rab 7	p = 0.0391 < α = 0.05	p = 0.5912 > α = 0.05	p = 0.6313 > α = 0.05	p = 0.0001 < α = 0.05
$Tr4_{55}$	Rab 5	p = 0.3295 > α = 0.05	p = 0.1915 > α = 0.05	p = 0.4456 > α = 0.05	p = 0.5218 > α = 0.05
	Rab 7	p = 0.0912 > α = 0.05	p = 0.7561 > α = 0.05	p = 0.8825 > α = 0.05	p = 0.8392 > α = 0.05
$Tr4_{77}$	Rab 5	p = 0.1539 > α = 0.05	p = 0.9386 > α = 0.05	p = 0.7286 > α = 0.05	p = 0.6762 > α = 0.05
	Rab 7	p = 0.0001 < α = 0.05	p = 0.9766 > α = 0.05	p = 0.1564 > α = 0.05	p = 0.1394 > α = 0.05

Table S9: Zeiss LSM 510 Meta microscope settings in Zen 2009 software.

Parameters	Values
Scaling X	0.070 µm
Scaling Y	0.070 µm
Scaling Z	0.140 µm
Image size	x: 1024, y: 1024, z: 29, channels: 4, 16-bit
Dimensions	x: 71.36 µm, Y: 71.36 µm, z: 3.92 µm
Scan mode	stack
Zoom	2.0
Objective	Plan-Apochromat 63x/1.4 Oil DIC
Pixel dwell	0.80 µs
Average	Line 4
Master gain	Ch4: 600 (for Alexa Fluor 555 channel) ChD: 311 (DIC) Ch3: 654 (for FITC channel) Ch2: 642 (for DAPI channel)
Digital gain	1.00
Digital offset	-0.11
Pinhole	100 µm
Filters	LP 560
Beam splitters	MBS : HFT UV/488/543/633 DBS1 : NFT 545 DBS3 : Mirror DBS2 : NFT 490
Lasers	* rhod for triple with fitc dapi 543 nm : 45.0 % * fitc for triple with dapi rhod 488 nm : 8.0 % * dapi for triple with rhod fitc 364 nm : 100.0 %

Additional Discussion

1. Concentric Nuclear Zones:

To further explain the idea of concentric nuclear zones introduced in this study, each of the 3D trafficking zones encompasses a volume-segment in 3D space surrounding the nucleus at varying distances (**Figure 4)**. The width of this imaginary volume-segment would correspond to the distance-range of a specified zone. This distance-range would be the distance from the surface of the nucleus, such that the surface contour of each zone (or a volume-segment) is the same as the surface contour of the nucleus. The zones closest to the nucleus (< 5 µm) were

considered ‘perinuclear zones’ because the trafficking pattern appeared to differ below this distance. This difference in trafficking pattern appeared to be mainly due to the higher probability for a number of polyplexes to exist in this region (< 5 μm) as compared to distances greater than 5 μm.

Interestingly, for $Tr4_{55}$ polyplexes, as the post-transfection time increased from 4 to 24 hours, the probability for the number of polyplexes to exist in the perinuclear zone of ~ 1 μm gradually increased from 0.27 to 0.54 (**Figure 5**). These results suggested that for $Tr4_{55}$ polyplexes, trafficking inside the inner zones (~1 μm) of the perinuclear region closer to nucleus occurred with a continually-increasing number of polyplexes gathering in the perinuclear region from 4 to 24 hours. A different trend was observed for polyplexes made with jetPEI™, Glycofect™ and $Tr4_{77}$, where it was found that the number of polyplexes in the perinuclear region decreased over 12 hours and then increased at the 24 hour time point. The decreased probability in the first 12 hours in the cases of polyplexes formed with jetPEI™, Glycofect™ and $Tr4_{77}$, may be indicative of a fraction of the polyplexes that were trafficking in and out of the perinuclear zones. Alternatively it may be indicative of an increasing number of pDNA complexes that may have entered the nucleus during these first 12 hours post transfection. Interestingly, however, we did not observe a significant increase in colocalization of pDNA complexes within the nucleus at 12 hours as compared to 4 hours due to high cell to cell variability.

SI Movie Caption

Movie S1: The movie depicts the spatial intracellular location of the $Tr4_{55}$ polyplexes containing FITC-labeled plasmid DNA (pseudo colored 'blue') in three dimensions (3D) at a time point of 24 hours post transfection. The nucleus is pseudo colored 'yellow' A snapshot of this image is also shown in **Figure 1** of the main manuscript. The movie is a compilation of over 500 individual images captured manually by rotating the volume in the Object Analyzer plugin in Huygens Essential Software (Scientific Volume Imaging SVI, Hilversum, The Netherlands).

Supplementary References

1. Fichter, K. M.; Ingle, N. P.; Mclendon, P. M.; Reineke, T. M., Polymeric Nucleic Acid Vehicles Exploit Active Interorganelle Trafficking Mechanisms. *ACS Nano* 2013, 7, 347-364.