Published in final edited form as:

Biotechnol Prog. 2015 November ; 31(6): 1685–1692. doi:10.1002/btpr.2146.

Correlating Intracellular Non-viral Polyplex Localization with Transfection Efficiency using High-Content Screening

Amaraporn Wongrakpanich¹, Meng Wu^{1,2}, and Aliasger K. Salem¹

¹Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, University of Iowa, Iowa City, IA, 52241

²The University of Iowa, High Throughput Screening Facility (UIHTS), College of Pharmacy, University of Iowa, Iowa City, IA, 52241

Abstract

High-content screening (HCS) has gained interest in cellular imaging because of its ability to provide statistically significant data from multiple parameters simultaneously in cell-based assays. Although HCS has been mainly used in drug discovery, it has other potentially useful applications, such as elucidating the processes involved in non-viral gene vector-mediated gene delivery, as was explored in this study. HCS was used to measure transfection efficiency and cytotoxicities of polyplexes made from fluorescently labeled polyethylenimine (PEI) and pDNA encoding EGFP (pEGFP-N1). The results generated using HCS were confirmed using more conventional and labor-intensive methods. For the first time, a relationship between transfected cells and the number of polyplexes in the cytoplasm was shown. Four to five polyplex signals were found in the cytoplasm of successfully transfected cells, whilst non-transfected cells harbored less than one polyplex signal within the cytoplasm. HCS has the potential to be used as a tool in the field gene delivery. HCS can not only simultaneously measure transfection efficiency and cytotoxicity of various non-viral gene vectors; it can also be used to track such vectors through various subcellular compartments.

Keywords

High-content screening; non-viral gene delivery; transfection; intracellular delivery; polyethylenimine; PEI

Introduction

High-content screening (HCS) is a combination of high-throughput techniques and fluorescent cellular imaging enabling simultaneous quantitative measurements of multiple parameters from one experiment. In addition, HCS collects a large amount of differential subcellular spatio-temporal information, providing unbiased statistically significant data, making it an excellent candidate for the intracellular tracking of submicron materials, such as DNA vectors^{1, 2}. Although, to date, HCS has been mainly focused on drug discovery^{2, 3},

it also has the potential to be of value in the gene delivery field. HCS can be used to assess the effect of non-viral gene vectors on cells *in vitro* where multiple parameters (such as cytotoxicity, transfection efficiency, cell permeability) can be rapidly and simultaneously measured ⁴.

Vectors that transport DNA/RNA into cells can be divided into two categories, viral and non-viral vectors. Viral vectors usually result in much higher transfection efficiencies when compared to non-viral vectors. However, their toxicity and immunogenicity issues are sometimes problematic and consequently need to be addressed or avoided ⁵. Non-viral gene delivery has gained substantial interest as a therapeutic tool because of its safety profile, ability to deliver large gene sizes, ease of preparation and its potential to be modified for cell- or tissue-targeting. These traits are generally considered strong advantages over current viral-based gene delivery systems. However, low transfection efficiencies are still a major concern for non-viral based gene delivery ⁶⁻⁹. To achieve high transfection efficiencies, the DNA, encoding the gene of interest, needs to be effectively taken up by cells and then transported to the nucleus ¹⁰. Cationic polymers such as polyethylenimine (PEI) and chitosan can form complexes with pDNA via electrostatic interactions which, when appropriately formulated, can create polyplexes ¹¹. To design a highly efficient gene vector, it is important to gain an insight into the mechanics and kinetics of uptake and intracellular trafficking pathways of gene vectors, DNA and polyplexes. Thus, the factors contributing to suboptimal transgene expression may be identified and potentially averted through subsequent modifications ¹².

Manifold efforts have been made to study intracellular trafficking processes and numerically quantify gene carriers within the cell and its subcellular compartments. For example, the importance of various uptake and trafficking pathways such as endocytosis and macropinocytosis have been assessed using conditions to specifically inhibit crucial steps in these pathways ¹². The internalization kinetics of single particles can be tracked using wide-field fluorescence microscopy in combination with custom-built software for single-particle tracking ¹³. Confocal microscopy and two-photon fluorescence correlation spectroscopy have also been used to track polyplexes ¹⁴⁻¹⁶. Among these studies, only Akita *et al.* have both quantified and localized the transfecting materials (using confocal image-assisted three-dimensionally-integrated quantification) ¹⁵. Although it is possible to observe the uptake and cellular trafficking of polyplexes by the aforementioned novel methodologies, they are limited by the number of cells that can be analyzed, thereby placing difficulties in accumulating sufficient data for statistical analysis.

In this study, we report on an application for HCS that involved evaluating the transfection efficiency and cytotoxicity of a commonly used non-viral gene vector, polyethylenimine (PEI). In addition, we show, for the first time, a relationship between successfully transfected cells and number of polyplexes or polyplex clusters inside the cytoplasm. This study demonstrates that HCS has the potential to be a powerful tool for analyzing uptake and intracellular trafficking of non-viral gene delivery vectors along with measuring other parameters, such as cytotoxicity and transfection efficiency, simultaneously.

Materials and methods

Cell lines and cell culture

Human Embryonic Kidney cells (HEK293) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1 mM Glutamax™ (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco) and 50 µg/ml gentamycin sulfate (Cellgro, Manassas, VA). Cells were maintained at 37°C and 5% CO₂.

Amplification and purification of pDNA

pEGFP-N1, a 4.7 kb plasmid encoding enhanced green fluorescent protein (GFP), was a generous gift from Satheesh Elangovan, College of Dentistry, University of Iowa. This pDNA was transformed into *Escherichia coli DH5α* and amplified in Lennox L Broth (Research Products International Corp., Mount Prospect, IL) media and purified using a GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, Co., St. Louis, MO). pDNA concentration in endotoxin-free water (Sigma-Aldrich) was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

Preparation of PEI polyplexes (PEIpp)

To be able to track the polyplexes, fluorescently tagged branched PEI was chosen. Rhodamine tagged branched polyethylenimine (rhPEI, MW 25kDa with a labeling ratio, Mol_{monomer}/Mol_{dye}, of 180/1) was purchased from Surflay Nanotech GmbH (Berlin, Germany).

PEI polyplexes (PEIpp) were formed based on the ionic interaction between positively charged PEI and negatively charged pDNA. PEIpp can be formed at varying ratios of amine groups in PEI to phosphate groups in pDNA (N/P ratios). In this study an N/P ratio of 10 was used. The rhPEI (240 µg/mL) and pDNA (100 µg/mL) solutions were prepared in UltraPure DNase/RNase-Free distilled water (Invitrogen, Grand Island, NY). All solutions were sterilized using 0.22 µm syringe filters (Millex®-GV, merck Millipore Ltd., Carrigtwohill, Germany). A volume of 125 µL of rhPEI solution was pipetted into 125 µL of pDNA solution, vortexed for 20 s and then incubated at room temperature for 30 min before use. The PEIpp were prepared fresh for each experiment. The final concentration of pDNA after the PEIpp were formed was 50 µg/mL.

Transfection and sample preparations for HCS

A solution of 0.01% poly-L-lysine (MW 150,000 – 300,000, Sigma-Aldrich) was used to coat the surface of the wells of the microplates (black 96-well cell carrier with transparent bottom, Perkin Elmer Inc., Waltham, MA) according to the manufacturer's protocol. HEK293 cells at a density of 5000 or 7500 cells per well were plated in a total volume of 100 µl of fully supplemented media. After 24 h, the media was aspirated and replaced with 100 µl of serum-free media containing PEIpp N/P 10 with pDNA amounts of 0.5 or 1 µg per well. An untreated group was used as a negative control. After cells were exposed to the PEIpp for 4 h, the media was aspirated and the cells were gently rinsed once with warm PBS

and then 100 μ l of fully supplemented media was added to each well. Twenty hours later media was removed and cells were gently rinsed once with PBS before being fixed with 4% formaldehyde (Alfa Aesar, Ward Hill, MA) in PBS for 30 min. Cells were then rinsed with PBS prior to incubating with 1 μ g/ml of Hoechst dye to stain the nucleus for at least 15 min prior to imaging.

Imaging using HCS and image analysis

The aforementioned microplates containing fixed and stained cells were loaded onto, and imaged with, a 20 $\times$ high NA objective on a High Content Screening Operetta[®] system (HCS, Perkin Elmer Inc. Waltham, MA). Three fluorescent channels of GFP (ex. at 460 – 490 nm, em. at 500 – 550 nm), Hoechst (ex. at 360 - 400 nm, em. at 410 - 480 nm) and rhodamine (ex. at 520 - 550nm, and em. at 560 - 630 nm) were utilized, with 18 fields/well and 8 stacks with 0.5 μ m intervals (Figure 1).

The images collected were analyzed by the accompanying Harmony[®] Image Analysis software (Perkin Elmer Inc., Waltham, MA). The image analysis was done with maximum projection of stack images, including: 1) Finding cells' nuclei using the Hoechst Channel. The nuclear areas of these cells were then identified as "true nuclei". After calculating the intensity of staining and morphology of the cells' nuclei, true nuclei were selected based on the following parameters: possessing areas smaller than 280 μ m²; having Hoechst intensities (mean, an average intensity from all pixels in the nucleus) larger than 2000; and having contrasts larger than 0.30. The contrast is the readout of intensity after normalization of the highest and lowest intensity to 1 and 0, respectively. 2) Finding cells using the GFP channel. By using the same method used for the calculation of intensity and morphology of the nuclei, the areas of the cells, or "true cells", were defined as follows: possessing areas smaller than 1040 μ m²; having GFP intensity (mean) larger than 0 (for both GFP(+) and GFP(–) cells); and having contrasts larger than 0.25. 3) Finding GFP(+) and GFP(–) cytosols using the GFP channel and true nuclei. The GFP(+) cytosols were selected with areas larger than 180 μ m² (calculated by subtracting the areas of the true nuclei from the areas of true cells) and GFP intensities larger than 120. The GFP(–) cytosols were selected similarly except that they possessed GFP intensities smaller than 120. 4) Finding the PEIpp, or "micronuclei", using the rhodamine channel, the cytosolic region or nuclear region. The PEIpp in the GFP(+) or GFP(–) cytosolic region were further defined by their particle area being smaller or equal to 80 μ m². In general, polyplexes made from branched PEI and pDNA at a N/P ratio of 10 contain sizes approximately 100 nm in diameter with a positive charge when measured in water at room temperature by dynamic light scattering¹⁷. It should be noted that the size of individual polyplexes are smaller than half of the wavelength of the excitation source ($1/2\lambda$: 260 – 275 nm) due to the light diffraction limit in our optical system. Thus, in the context of the HCS analysis here, PEI polyplexes or PEIpp may be referring to single PEI polyplex particles or a cluster of PEI polyplexes. In the analysis, the true nuclei values were used to define viable cell numbers per well, and the percentage of GFP(+) cells of the total cell population (both GFP(+) and GFP(–) cells) was used to define the transfection efficiency.

Cytotoxicity of PEIpp using MTS assay

Cytotoxicity of PEIpp was determined using an MTS assay. HEK293 cells were plated into wells of a poly-l-lysine coated 96-well plate at two different seeding densities, 5000 and 7500 cells per well. Twenty-four hours later, media was aspirated and replaced with PEIpp N/P 10 (containing either 0.5 or 1 μ g of pDNA) in serum free media. Cells were exposed to treatments for 4 h, rinsed gently with warm PBS and then complete DMEM media was added. After 24 h, media was aspirated and cells were rinsed gently with PBS before being replaced with 100 μ L of fresh media plus 20 μ L of MTS tetrazolium compound (CellTiter 96[®] AQueous One Solution, Promega Corporation, Madison, WI). The plate was incubated at 37°C and 5% CO₂ for 4 h and then the absorbance of the solution in each well was recorded at 490 nm using a Spectra Max plus 384 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The background of 490 nm absorbance was corrected by subtracting absorbance obtained from the experimental well with absorbance obtained from 100 μ L of media and 20 μ L of MTS tetrazolium compound. Percent relative cell viability values were determined by dividing the absorbance obtained from wells containing treated cells by absorbance obtained from untreated cells with the same seeding cell density (5000 or 7500 cells per well) and multiplying the resultant value by a factor of 100.

Statistical analysis

Data are expressed as mean $\pm$ SEM. To analyze GFP expression in cells with 0.5 μ g and 1.0 μ g of pDNA in PEIpp, One-way ANOVA with Tukey's multiple comparisons test was performed. To analyze the number of PEIpp in cytoplasm, Two-way ANOVA with Bonferroni's multiple comparisons test was used. To analyze cell numbers, Two-Way ANOVA with Dunnett's multiple comparisons test was performed. All statistical analyses were conducted using GraphPad Prism 6 for Windows (GraphPad Software, Inc., San Diego, CA, www.graphpad.com). The *p*-values of less than 0.05 were considered significant.

Results and Discussion

HCS can rapidly determine transfection efficiencies

As a proof of concept, transfection of HEK293 cells with PEIpp was performed in these studies. PEI is a well-established cationic polymer used for non-viral gene delivery¹⁸⁻²³. The HEK293 cell line was chosen because it is widely used in transient gene expression systems²⁴. As described in the methods section, HEK293 cells were exposed to PEIpp complexes (containing pEGFPN-1) and subsequently (24 hours later) imaged using HCS. In order to determine transfection efficiency, the fluorescence intensity obtained from each cell was determined. By contrasting untreated/mock treated cells (negative controls) and GFP transfected cells, a threshold (mean intensity of the cell) of 120 (relative fluorescence intensity) was established such that there were virtually no GFP(+) cells in the mock transfected cells, and any cells with a relative fluorescence intensity above the threshold of 120 in the pEGFPN-1 transfected cells were considered GFP(+). The ratio of GFP(+) cells to total cells was used to define the transfection efficiency. In Figure 2A, an image generated by the HCS imaging system shows GFP(+) cells as green and cells expressing undetectable levels of GFP (GFP(-)) as red. Figure 2B shows untreated cells with no GFP expression (all

red). It was determined that PEIpp had transfection efficiencies of 4.0% and 8.9% with 0.5 μ g and 1.0 μ g of pDNA per well, respectively (Figure 3A). Increasing the amount of pDNA per well from 0.5 μ g to 1.0 μ g significantly increased the transfection efficiency (p -value < 0.01). In comparison, manual counting of GFP(+) and GFP(–) cells (at least 900 cells per well) from the same set of images was performed and showed similar results in terms of transfection efficiencies to those obtained using HCS. In figure 3B, manual counting showed that PEIpp had transfection efficiencies of 5.2% and 8.0% with 0.5 μ g and 1.0 μ g of pDNA per well, respectively. This manual counting is a validation for using HCS as a reliable tool for measuring transfection efficiencies when the transgene product is inherently detectable or rendered detectable through fluorescence. To provide statistically significant data, large sample sizes are needed. As the sample size (cell number) increases, the time and labor used to manually gather the data proportionally increases. The results from HCS were automatically obtained from confocal images that consisted of between 1000 – 8000 cells per well. Thus, HCS provides an opportunity to reduce bias and analyze a larger data set compared to manual counting which requires tedious labor and inordinately longer times particular when multiple parameters are being acquired.

Cell enumeration using HCS as an indicator of cytotoxicity

In addition to measuring the transfection efficiency, HCS can also count the cells simultaneously for cytotoxicity evaluation. Using Hoechst dye stained nuclei as a marker for cells, it was possible to program the HCS software to identify and enumerate the number of cells in each well (Figure 4). An algorithm in the HCS software was set to exclude dying (or dead) cells containing fragmented nuclei from the analysis. Only cells containing intact nuclei were enumerated. To validate the reliability of HCS in measuring the cytotoxicity of PEIpp an MTS assay was run in parallel. The MTS assay is a well-known colorimetric assay for measuring cell cytotoxicity²⁵. The results obtained from this assay were expressed as percentage relative cell viability compared to the untreated cells.

Viable cell numbers per well obtained from HCS (Figure 5A) were compared to results obtained from the MTS assay (Figure 5B) and were found to be comparable. Wells containing cells that were treated with higher amounts of PEIpp (1.0 μ g pDNA per well) possessed lower cell numbers, as determined by HCS, compared to wells treated with PEIpp containing 0.5 μ g pDNA. The findings were corroborated by results generated from the MTS assay performed in parallel. Moreover, HCS provided, in contrast to the MTS assay, absolute viable cell numbers per well.

Thus, HCS, along with being capable of accurately measuring transfection efficiency (shown above), can simultaneously be used to measure cytotoxicity of non-viral gene delivery systems.

GFP(+) cells possessed a higher number of PEIpp in the cytoplasm than GFP(–) cells

Using HCS, the cytoplasmic area of each cell (or region of interest) was identified using endogenous autofluorescence (Figure 6B). The cytoplasmic region was programmed to be delineated from the nuclear region by differential fluorescence detection using Hoechst stain. Using HCS, rhodamine-labeled PEIpp were detected and enumerated in the cytoplasm

and the nucleus in both GFP(+) and GFP(-) cells (Figure 6C). For the first time, we showed a relationship between successfully transfected cells and the number of PEIpp that can be quantified. Whilst no PEIpp were detected in the nuclei of either GFP(+) or GFP(-) cells, approximately 4-5 polyplexes/cell were detected in the cytoplasm of GFP(+) cells that were treated with PEIpp delivering 0.5 or 1.0 μ g pDNA per well. This was found to be significantly (p -value < 0.0001) higher than the number of PEIpp found in the cytoplasm of GFP(-) cells (less than 1 complex/cell, on average). Although cells that were treated with PEIpp (1.0 μ g pDNA per well) showed a significantly higher transfection efficiency than cells that were treated with PEIpp (0.5 μ g pDNA per well) (Figure 3B), there was no significant difference in the number of PEIpp per cell when 0.5 μ g versus 1 μ g of pDNA was compared in the GFP(+) populations (Figure 7). Since PEIpp toxicity increases with the dose of PEIpp, it is possible that cells that contained more than 4 – 5 PEIpp could not survive long enough to be detected at the time of data acquisition (24 hours post-transfection). It is possible that 4 - 5 polyplexes in the cytoplasm is an optimal amount for the successful transfection of healthy cells.

In this study, PEIpp were not detected inside the nuclei of HEK293 cells at 24 hours post-transfection. This result suggests that pDNA may disassociate from the PEIpp in the cytoplasm prior to reaching the nucleus, where it is then available for transcription. Support for this idea comes from Remy-Kristensen et al.²⁶ who studied the role of PEIpp endocytosis in mouse fibroblasts (L929) using confocal microscopy. Ninety minutes post-transfection, labeled PEIpp could not be detected inside the transfected L929 nucleus but instead were found on the outer nuclear membrane surface. In addition, Itaka et al.²⁷ studied intracellular distribution of PEIpp using confocal microscopy with fluorescence resonance energy transfer in HEK293T cells (a highly transfectable sub-clone of HEK293). Twenty-four hours after transfection, PEIpp were distributed in the cytoplasm but there was no evidence of PEIpp in the nuclei. In contrast, Godbey et al.¹⁴ studied PEIpp in EA.hy 926 cells (derived from a fusion of human lung carcinoma, A549, and human umbilical vein endothelial cells) using confocal microscopy. PEIpp were localized in nuclei after 3.5-4.5 hours post-transfection. However, to the best of our knowledge this latter finding has not been reproduced in other cell types and may reflect an idiosyncratic trait that pertains to this particular cell line.

The purpose of this study was to demonstrate the potential of using HCS as a tool to study non-viral gene vector-mediated transfection. Specifically, regarding PEIpp, there is much interest in enhancing the transfection efficiency of these polyplexes whilst simultaneously reducing their cytotoxicity. Thus, studies of mechanism of transfection by these polyplexes are believed to be important^{28, 29}. Since HCS is an image based system, it is feasible to track non-viral vectors and pDNAs by labeling them with fluorescent tags. Because of its automation, HCS has the potential to be used to elucidate optimal conditions achieving maximal transgene expression with minimum toxicity for a variety of gene vectors using various cell types under a wide range of conditions^{30, 31}. Conventional modes of analysis (manual counting and MTS assays) were used to validate the HCS used here, thereby establishing the accuracy of HCS⁴. Combined with providing large data sets, HCS also has the ability to study polyplexes using live cell imaging and thus collect data at different time

points which could be used to elucidate the steps of intracellular trafficking of non-viral based gene carriers.

Conclusion

The capacity of HCS to make quantitative multi-parametric measurements of cells and cell populations through rapid automated fluorescence image capturing has been of great value in drug discovery. HCS, however, also has great promise in the gene delivery field, where not only information about transfection efficiencies and cytotoxicities of various non-viral gene vectors can be accrued, but also, as shown here, the tracking of vectors (such as PEIpp) inside cells can be simultaneously achievable. This provides a strong rationale for using HCS in the future as a screening system for tens or hundreds of novel transfection reagents simultaneously, so long as transgenes expressing fluorescent proteins are used for determining transfection efficiencies, and fluorescently tagged vectors are used for establishing the mechanism(s) of transfection through subcellular tracking.

Acknowledgements

We gratefully acknowledge support from the National Cancer Institute at the National Institutes of Health (P50 CA97274/ P30 CA086862 Cancer Center Support Grant) and the Lyle and Sharon Bighley Professorship. A. Wongrakpanich gratefully acknowledges support from The Royal Thai Government Scholarship. We thank Dr. Sean M. Geary for expert reading of the manuscript.

Notation

Ex.	Excitation
Em.	Emission
HCS	High-content screening
pEGFP-N1	pDNA encoding enhanced green fluorescent protein
PEI	Polyethylenimine
PEIpp	Polyethylenimine polyplexes (polyethylenimine complexes with pDNA)
pp	Polyplexes
rhPEI	Rhodamine tagged branched polyethylenimine

References

1. Zanella F, Lorens JB, Link W. High content screening: seeing is believing. Trends in biotechnology. 2010; 28(5):237–45. [PubMed: 20346526]
2. Buchser, W.; Collins, M.; Garyantes, T.; Guha, R.; Haney, S.; Lemmon, V.; Li, Z.; Trask, OJ. Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging.. In: Sittampalam, GS.; Coussens, NP.; Nelson, H.; Arkin, M.; Auld, D.; Austin, C.; Bejcek, B.; Glicksman, M.; Inglese, J.; Lemmon, V.; Li, Z.; McGee, J.; McManus, O.; Minor, L.; Napper, A.; Riss, T.; Trask, OJ.; Weidner, J., editors. Assay Guidance Manual. Bethesda (MD): 2004.
3. Giuliano KA, Haskins JR, Taylor DL. Advances in high content screening for drug discovery. Assay and drug development technologies. 2003; 1(4):565–77. [PubMed: 15090253]

4. de Raad M, Teunissen EA, Lelieveld D, Egan DA, Mastrobattista E. High-content screening of peptide-based non-viral gene delivery systems. *Journal of controlled release : official journal of the Controlled Release Society*. 2012; 158(3):433–42. [PubMed: 21983020]
5. Ramamoorth M, Narvekar A. Non viral vectors in gene therapy- an overview. *Journal of clinical and diagnostic research : JCDR*. 2015; 9(1):Ge01–6. [PubMed: 25738007]
6. Ojea-Jimenez I, Tort O, Lorenzo J, Puentes VF. Engineered nonviral nanocarriers for intracellular gene delivery applications. *Biomedical materials (Bristol, England)*. 2012; 7(5):054106.
7. Wang W, Li W, Ma N, Steinhoff G. Non-viral gene delivery methods. *Current pharmaceutical biotechnology*. 2013; 14(1):46–60. [PubMed: 23437936]
8. Wang D, Gao G. State-of-the-art human gene therapy: part I. Gene delivery technologies. *Discovery medicine*. 2014; 18(97):67–77. [PubMed: 25091489]
9. Jin S, Ye K. Nanoparticle-mediated drug delivery and gene therapy. *Biotechnology progress*. 2007; 23(1):32–41. [PubMed: 17269667]
10. Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nature biotechnology*. 2000; 18(1):33–7.
11. Midoux P, Breuzard G, Gomez JP, Pichon C. Polymer-based gene delivery: a current review on the uptake and intracellular trafficking of polyplexes. *Current gene therapy*. 2008; 8(5):335–52. [PubMed: 18855631]
12. Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacological reviews*. 2006; 58(1):32–45. [PubMed: 16507881]
13. de Bruin K, Ruthardt N, von Gersdorff K, Bausinger R, Wagner E, Ogris M, Brauchle C. Cellular dynamics of EGF receptor-targeted synthetic viruses. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2007; 15(7):1297–305. [PubMed: 17457321]
14. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96(9):5177–8. [PubMed: 10220439]
15. Akita H, Ito R, Khalil IA, Futaki S, Harashima H. Quantitative three-dimensional analysis of the intracellular trafficking of plasmid DNA transfected by a nonviral gene delivery system using confocal laser scanning microscopy. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2004; 9(3):443–51. [PubMed: 15006612]
16. Clamme JP, Krishnamoorthy G, Mely Y. Intracellular dynamics of the gene delivery vehicle polyethylenimine during transfection: investigation by two-photon fluorescence correlation spectroscopy. *Biochimica et biophysica acta*. 2003; 1617(1-2):52–61. [PubMed: 14637019]
17. D'Mello S, Salem AK, Hong L, Elangovan S. Characterization and evaluation of the efficacy of cationic complex mediated plasmid DNA delivery in human embryonic palatal mesenchyme cells. *J Tissue Eng Regen Med*. 2014
18. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92(16):7297–301. [PubMed: 7638184]
19. Intra J, Salem AK. Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo. *J Control Release*. 2008; 130(2):129–138. [PubMed: 18538436]
20. Zhang XQ, Intra J, Salem AK. Comparative study of poly (lactic-co-glycolic acid)-poly ethyleneimine-plasmid DNA microparticles prepared using double emulsion methods. *Journal of Microencapsulation*. 2008; 25(1):1–12. [PubMed: 18188727]
21. Jiang DH, Salem AK. Optimized dextran-polyethylenimine conjugates are efficient non-viral vectors with reduced cytotoxicity when used in serum containing environments. *Int J Pharmaceut*. 2012; 427(1):71–79.
22. Kim N, Jiang DH, Jacobi AM, Lennox KA, Rose SD, Behlke MA, Salem AK. Synthesis and characterization of mannosylated pegylated polyethylenimine as a carrier for siRNA. *Int J Pharmaceut*. 2012; 427(1):123–133.
23. Elangovan S, D'Mello SR, Hong L, Ross RD, Allamargot C, Dawson DV, Stanford CM, Johnson GK, Sumner DR, Salem AK. The enhancement of bone regeneration by gene activated matrix

- encoding for platelet derived growth factor. *Biomaterials*. 2014; 35(2):737–747. [PubMed: 24161167]
24. Thomas P, Smart TG. HEK293 cell line: a vehicle for the expression of recombinant proteins. *Journal of pharmacological and toxicological methods*. 2005; 51(3):187–200. [PubMed: 15862464]
25. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology annual review*. 2005; 11:127–52
26. Remy-Kristensen A, Clamme JP, Vuilleumier C, Kuhry JG, Mely Y. Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes. *Biochimica et biophysica acta*. 2001; 1514(1):21–32. [PubMed: 11513802]
27. Itaka K, Harada A, Yamasaki Y, Nakamura K, Kawaguchi H, Kataoka K. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. *The journal of gene medicine*. 2004; 6(1):76–84. [PubMed: 14716679]
28. Rajendra Y, Kiseljak D, Baldi L, Wurm FM, Hacker DL. Transcriptional and post-transcriptional limitations of high-yielding, PEI-mediated transient transfection with CHO and HEK-293E cells. *Biotechnology progress*. 2015; 31(2):541–9. [PubMed: 25683738]
29. Mozley OL, Thompson BC, Fernandez-Martell A, James DC. A mechanistic dissection of polyethylenimine mediated transfection of CHO cells: to enhance the efficiency of recombinant DNA utilization. *Biotechnology progress*. 2014; 30(5):1161–70. [PubMed: 24850296]
30. Liu C, Dalby B, Chen W, Kilzer JM, Chiou HC. Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. *Molecular biotechnology*. 2008; 39(2):141–53. [PubMed: 18327552]
31. Subrizi A, Yliperttula M, Tibaldi L, Schacht E, Dubruel P, Joliot A, Urtti A. Optimized transfection protocol for efficient in vitro non-viral polymeric gene delivery to human retinal pigment epithelial cells (ARPE-19). 2009

Figure 1.
An example of (A) the 18-fields realistic view obtained from 1 well in 96-well plate and (B) the 18-fields well packed view. Scale bars in both images represent 1 mm.

Figure 2.

Images showing GFP(+) (green) and GFP(–) (red) cells from (A) a culture of HEK293 cells that had been treated with PEIpp (containing pEGFP-N1) and (B) an untreated culture of HEK293 cells. Designation of GFP(+) versus GFP(–) was based on cell fluorescence intensity above and below a set threshold, respectively(see methods section for details). All scale bars represent 100 μm.

Figure 3. Percentage GFP(+) cells in HEK293 cultures treated with PEIpp (0.5 and 1.0 µg of pDNA per well) as determined by (A) HCS and (B) manual counting. Data are presented as mean $\pm$ SEM (n = 3 - 4). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. 0.5 µg and 1.0 µg represent the amount of pDNA in each well.

Figure 4. Nuclei stained by Hoechst dye were used to determine cell number from wells seeded with (A) 5000 cells and (B) 7500 cells. (C) The nuclei were counted as shown using different colored cell perimeters. These different colors allowed adjacent nuclei to be distinguished. Scale bars in all images represent 100 μm .

Figure 5. Cell cytotoxicity as measured by (A) numbers of cells per well as defined by HCS and (B) percentage relative cell viability obtained from MTS assay. Percentage relative cell viability values were determined as described in the methods section. Data are presented as mean $\pm$ SEM ($n = 3 - 4$:A, $n = 5$:B). **** $p < 0.0001$, ** $p < 0.01$. 0.5 μg and 1.0 μg represent the amount of pDNA in each well.

Figure 6.

Images showing: (A) GFP expression (green) in HEK293 cells (nuclei are blue) with PEIpp (yellow); (B) the cytoplasmic region as determined through computer programmed software designed to delineate, through differential fluorescence detection, the nucleus (stained with Hoechst stain) from the cytoplasm (detected through expression of endogenous autofluorescence); (C) the presence of PEIpp (indicated here as multicolored dots) in the cytoplasm. Scale bars in both images represent 100 μm .

Figure 7. Number of PEIpp in cytoplasmic region of GFP(+) and GFP(-) cell populations, as calculated using HCS. Data are presented as mean $\pm$ SEM ($n = 3$). **** $p < 0.0001$. 0.5 μg and 1.0 μg represent the amount of pDNA in each well.