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Quantification of Plasmid DNA Copies in the Nucleus after Lipoplex and Polyplex Transfection

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Abstract

Nuclear uptake of plasmid DNA is one of the many cellular barriers that limit the efficiency of non-viral gene delivery systems. We have determined the number of plasmids that reach the nucleus of a transfected cell using an internally standardized quantitative PCR (qPCR) assay. We isolated nuclei using two different protocols: a density gradient technique and a detergent-based method. The density gradient procedure yielded nuclei with substantially less adhering plasmids on the outside of the nuclei. Using the density gradient protocol we determined that cells transfected with LipofectamineTM lipoplexes or polyethylenimine polyplexes contained between 75 and 50,000 plasmids/nucleus, depending on the applied plasmid dose. Any increase above 3000 plasmids/nucleus resulted in only marginal increases in transgene expression. Furthermore, lipoplex-delivered plasmids were more efficiently expressed, on the basis of protein expression per plasmid number in the nucleus, than polyplex-delivered plasmids. This indicates that polymer may remain bound to some plasmids in the nucleus. Lastly, by sorting transfected cells into high- and low-expressing sub-populations, we observe that a sub-population of cells contain 3x greater plasmids/nucleus but express nearly 100x more transgene than other cells within a single transfection reaction. Taken together these results suggest the importance of considering the processes downstream from nuclear entry for strategies to improve the efficiency of gene transfer reagents.

Keywords

cell fractionation; drug delivery; gene therapy; intracellular trafficking; iodixanol

INTRODUCTION

Despite many advances in the development of nonviral vectors over the past 25 years, nonviral gene therapy has not been able to achieve the gene transfer efficiency of its viral counterparts. However, the hope for reduced toxicity and improved ease of preparation compared to viruses has continued to motivate the design and study of new polymers, lipids, peptides, and physical methods that can efficiently deliver DNA *in vivo*. The more promising new methods will be

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devised by logical consideration of the many barriers that the delivered DNA must traverse in its journey from the outside of the body to the nucleus of target cells. Examples of strategies that overcome these barriers include PEGylation to improve circulation time [1,2], targeting agents for internalization into specific cell types [3–6], pH-responsive agents that can disrupt endosomes to allow release of DNA into the cytoplasm [1,4,7], motor-protein-binding peptides that can allow for active transport of DNA towards the nucleus [8,9], and nuclear localization signal peptides that may trigger import of associated cargo into the nucleus [10].

In addition to new techniques to overcome cellular barriers, the development of gene therapy vectors will also benefit greatly from an improved understanding of the intracellular events that occur during gene transfer [11]. The aim of this study is to evaluate nuclei isolation techniques and apply PCR methodologies to quantify the number of plasmids in the nuclei isolated from cells transfected under different conditions. Unlike most drugs, whose cellular targets are on the surface of the cell membrane, DNA drugs used for gene therapy must reach the nucleus in the interior of the cell to take action. A metric for the amount of DNA that enters the nucleus will allow for the determination of the minimum and optimum amount of nuclear-delivered DNA required for detectable and best possible transgene expression, respectively, and for the comparison of the nuclear delivery efficiency of various gene transfer strategies.

Advances in fluorescent labeling of both DNA and carriers molecules has allowed for intracellular visualization of nonviral (and viral) vectors in live cells. For example, an elegant recent study using quantum-dot fluorescence resonance energy transfer (QD-FRET) was able to determine the relative intracellular stability of various polymer/DNA complexes as well as the kinetics and location of DNA release from those complexes [12]. It is important to consider however that these fluorescent experiments require modification of the delivered DNA that could alter their intracellular trafficking and should be complemented with experiments that do not require labeling. This is particularly important for quantitative experiments in which it is difficult to ensure that the fluorescent signal has not been cleaved from the DNA or carrier. In the present work, we utilize a detergent-free subcellular fractionation technique [13] to isolate nuclei from transfected cells with minimal extranuclear contamination and quantify the amount of unlabeled plasmid per isolated nuclei with an internally standardized relative quantification qPCR assay. Using this approach, we compare the nuclear delivery efficiency of two commonly used nonviral gene transfer agents, PEI and Lipofectamine and also investigate the relationship between expression and intranuclear plasmid within a population of transfected cells.

MATERIALS AND METHODS

Materials

High molecular weight (25 kD) polyethylenimine (PEI) was purchased from Aldrich (Milwaukee, WI) diluted to 100 mg/ml in 1 M NaCl and dialyzed in Spectra/Por 4 (2.5 cm, 12–14 kD molecular weight cut-off) cellulose dialysis tubing (Spectrum Labs, Rancho Dominguez, CA) against 100 volumes of: 1 M NaCl, then 100 mM Hepes pH 7.4, then deionized water twice. The PEI was then lyophilized and resuspended in distilled water. Lipofectamine was purchased from Invitrogen (Carlsbad, CA). pCMV-GFP (pGFP, 3831 bp) and pCMV-luciferase (pLuc, 4739 bp) plasmids were generous gifts from Valentis (Burlingame, CA). Iodixanol (60% Optiprep Density Gradient Medium) was obtained from Sigma (St. Louis, MO).

Cell culture and Transfection

B16F10 mouse melanoma cells or A549 human lung carcinoma cells were seeded in T-75 culture dishes at 1.8×10^6 and 2.5×10^6 cells in MEM Eagle's with Earle's BSS containing

10% FBS, 1% Sodium pyruvate, 1% non-essential amino acids and RPMI containing 10% FBS, respectively, and incubated for 24 hours at 37°C, 5% CO₂. For PEI transfection, polyplexes were prepared by combining PEI and plasmid DNA at a 1:1.3 (w/w) ratio in HEPES buffer (10 mM HEPES, 140 mM NaCl) and incubating at room temperature for 30 minutes. Polyplexes were then diluted into media with serum to produce desired concentration in a total volume of 5 ml and incubated with the cells at 37°C, 5% CO₂ for 3 hours. Lipofectamine (LFN) transfection was performed according to the manufacturer's instructions. Briefly, lipoplexes were prepared by combining 160 µl LFN Plus reagent with 16 µg of plasmid DNA suspended in 1 ml of serum-free media and incubated for 15 minutes at room temperature. This solution was then combined with 40 µl of LFN in 1 ml serum-free media and further incubated for 15 minutes at room temperature. Lipoplexes were then diluted into serum-free media to produce desired concentration in a total volume of 5 ml and incubated with the cells at 37°C, 5% CO₂ for 3 hours. Following removal of polyplexes or lipoplexes from the cells and an additional 21 hour incubation at 37°C, 5% CO₂ in fresh media with serum, cells were harvested for cell sorting on a MoFlo high-speed cell sorter (Cytomation, Ft. Collins, CO) when indicated or directly for nuclei isolation, as described below.

Isolation of nuclei from mammalian cells

Nuclei were isolated from A549 or B16F10 cells by three different methods (Fig. 1a). (1) Detergent method: Adherent cells were washed with PBS, without calcium or magnesium (PBS) incubated with lysis buffer (0.5% Nonidet p-40, 10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂) for 30 seconds at room temperature to remove cell membranes, and washed twice with PBS. Nuclei remaining on the culture dish were then scrapped off of the surface using a cell scraper (Fisher Scientific, Pittsburgh, PA), pelleted by centrifuging at 1400 × g for 5 minutes, washed twice in PBS, and resuspended in 1 ml of lysis buffer. (2) Iodixanol method: Adherent cells were washed with PBS, trypsinized by incubating with 0.05% trypsin with EDTA in Saline A (UCSF Cell Culture Facility, San Francisco, CA) for 1–2 minutes at 37 °C, pelleted by centrifuging at 1400 × g for 5 minutes, washed twice with PBS and resuspended in 1 ml nuclei buffer (10 mM PIPES pH 7.4, 1mM DTT, 2 mM MgCl₂, 10 mM KCl.). After a 25 minute incubation of the cells in the nuclei buffer, the cells were lysed in a dounce homogenizer with 50 strokes of the tight pestle. Cell lysis was confirmed by observation of cells with Trypan Blue. A discontinuous iodixanol gradient was prepared by underlaying 3 ml of 30% and 35% iodixanol in isotonic buffer (130 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, pH 7.4). Cell lysates were mixed with an equal volume of 50% iodixanol in isotonic buffer to make a 25% solution, layered on top of the 30/35% iodixanol gradient, and spun in a swinging-bucket ultracentrifuge (Beckman Coulter, Fullerton, CA) for 20 minutes at 10,000 × g. Nuclei were recovered from the 25/30% iodixanol interface by making a hole in the bottom of the ultracentrifuge tube with a 16-gauge needle and collecting fractions into ultracentrifuge tubes. (3) Combination method: Nuclei prepared by the detergent method (#1 above) were mixed with an equal volume of 50% iodixanol in isotonic buffer to make a 25% iodixanol solution, layered on top of the 30/35% iodixanol gradient, and ultracentrifuged and recovered as in the iodixanol method (#2 above). Following all methods, isolated nuclei were either prepared for washing and/or total DNA extraction or were stored at –20°C.

Confocal Microscopy

pLuc was labeled with Cy5 using the LabelIt kit (Mirus, Madison, WI) following the manufacturer's instructions. The density of labeling was determined to be 84 Cy5 molecules per plasmid. Rhodamine-labeled PEI was prepared by incubating 5(6)-tetramethylrhodamine isothiocyanate (Research Organics, Cleveland, OH) with PEI at a 2/1 molar ratio in dichloromethane/ethanol for 3 hours at 37°C, evaporating off the organic solvent, redissolving in 1 M NaCl, and dialyzing against three changes of 50 mM HEPES, 100 mM NaCl, pH 7.4. Cy5-pLuc/Rhodamine-PEI polyplexes and Cy5-pLuc/LFN lipoplexes were prepared and

transfected into B16F10 cells in the same manner as described above. Following transfection and nuclear isolation, nuclei were pipetted onto a coverslip and viewed on a Bio-Rad 600 confocal scanning laser microscope (Bio-Rad, Hercules, CA) using LaserSharp Software. One hundred iodixanol nuclei and at least 400 detergent nuclei were counted for each transfection reagent to determine the percentage of nuclei that occur in clumps (three or more nuclei) and contain extranuclear DNA.

Nuclei washing

To prepare nuclei with plasmid DNA only on their exterior, detergent nuclei from untransfected cells were incubated with an excess of pLuc plasmid or PEI/pLuc polyplexes for 1 hour at 4°C and washed with lysis buffer. To test conditions for washing nuclei to remove extranuclear DNA, plasmid-coated nuclei were incubated with 25 mM spermine, 100 µg/ml poly-aspartic acid (pASPA), or 150 µg/ml βgal plasmid for 1 hour on ice and washed twice with lysis buffer. Alternatively, plasmid- or polyplex-coated nuclei were incubated with a restriction enzyme (1 unit/5000–10000 nuclei) for 3 hours at 37°C with shaking followed by a 30 minute incubation at 65°C to inactivate the enzyme.

DNA extraction

Isolated nuclei were lysed by incubating them in 0.5% SDS, 100 µg/ml proteinase K, and 20 µg/ml DNase-free RNase for 4–5 hours at 50°C. Total DNA was then isolated from nuclear lysates by one extraction in one volume of TE-saturated phenol (Invitrogen, Carlsbad, CA) and two extractions each in one volume of 25:24:1 phenol/chloroform/isoamyl alcohol (Invitrogen, Carlsbad, CA). Following two additional washes with water-saturated ether, total DNA was precipitated with 3 volumes of 95% ethanol and 0.1 volumes of 3M sodium acetate for 20 minutes at –70°C or overnight at –20°C, washed with 70% ethanol, and stored in TE buffer (Qiagen, Valencia, CA) at 4°C.

Relative qPCR assay for measuring plasmids per nucleus

The relative amount of pLuc plasmid, genomic actin sequence, and genomic gapdh sequence were determined in a relative quantification real-time PCR assay. The primers and probes used for luciferase were synthesized by Integrated DNA Technologies (Coralville, IA) and have the following sequences- forward primer (LucF): GTACACGTTTCGTCACATCTC, reverse primer (LucR): TTAGGCAGACCAGTAGATCC, and probe (LucProbe): CGATTTTGTGCCAGAGTCCTTCG. LucProbe was modified with fluorescein (6-FAM) on its 5' end and Black Hole Quencher® 1 (BHQ-1) on its 3' end. The luciferase amplicon is 126 bp long. The primers and probes for actin and gapdh were purchased from Applied Biosystems (Foster City, CA) as the following kits- Mouse ACTB Endogenous Control for mouse actin (115 bp amplicon); Mouse GAPD Endogenous Control for mouse gapdh (107 bp amplicon); Taqman® Gene Expression Assay Hs03023880_g1 for human actin (139 bp amplicon); and Taqman® Gene Expression Assay Hs02786624_g1 for human gapdh (157 bp amplicon). Each of these kits contain a FAM-labeled TaqMan® MGB probe and were selected based on their ability to detect genomic DNA.

The following reagents were used for luciferase amplification in 20 µl: 1 µL of DNA (1–5 ng), 500 nM of LucF, 500 nM of LucR, 250 nM of LucProbe, 10 µL of 2X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and the final volume was adjusted with sterile water. The following reagents were used for actin and gapdh amplification in 20 µl: 1 µL of DNA (1–5 ng), 1 µl of 20x appropriate kit (final concentrations: 900 nM of each primer, 250 nM of probe), 10 µL of 2X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and the final volume was adjusted with sterile water. The thermal cycling conditions were 10 minutes at 95°C followed by 50 cycles at 95°C for 15 seconds and

60°C for 1 minute. The PCR was performed in an Applied Biosystems (Foster City, CA) 384-well plate 7900HT Real-Time PCR System.

To calibrate the PCR system, a standard curve of known amounts of the luciferase plasmid (pLuc), a plasmid containing the mouse or human β -actin sequence (pAct), and a plasmid containing the mouse or human gapdh sequence (pGap) were prepared and run each time on the same reaction plate as test samples. Human and mouse pAct and pGap plasmids were purchased as I.M.A.G.E. clones from Invitrogen (Carlsbad, CA) and purified with a HiSpeed Plasmid Midi Kit (Qiagen, Valencia, CA). The specific I.M.A.G.E. clones used, selected because they contain the same portion of the genomic gene that is amplified by the PCR kits, were as follows: mouse actin-3995253, human actin-3451917, mouse gapdh-2655526, human gapdh-5497708. The linear range of Ct versus the logarithm of the template plasmid amount in the qPCR system was at least between 10^3 and 10^7 plasmids and the PCR efficiency (determined by the formula, efficiency = $10^{-(1/\text{slope})}-1$) was similar for all three genes (Fig. 1b). The difference in Ct values between luciferase and actin (Fig. 1b, inset), luciferase and gapdh (not shown), and actin and gapdh (not shown) were also plotted against the logarithm of the template plasmid amount and the slope of the regression lines through these data were < 0.1 , further indicating that the amplification efficiencies are comparable. A linear curve with extracted genomic DNA as the template revealed a linear range of at least 0.1 to 10 ng of DNA for actin and gapdh detection with PCR efficiencies similar (within 2%) to those for pAct and pGap, respectively (not shown). Using the standard curve of pAct directly to quantify the number of actin copies in a sample of extracted genomic DNA revealed approximately 2 copies/genome/6.6 pg of DNA extracted from both iodixanol and detergent nuclei (not shown), which we would expect for a single-copy gene.

The standard curves of plasmids allowed for a measure of the ΔCt for a known ratio of the two genes (1:1). The ratio of luciferase (L) to actin (A) in the test samples, for example, could then be determined by measuring the ΔCt for each and using the formula $L/A = 2^{-\Delta\Delta\text{Ct}}$ where the $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{samples}} - \Delta\text{Ct}_{\text{calibrators}}$ [14]. Plasmids/nuclei (P/N) can then be calculated as $P/N = 2 * L/A$, since each plasmid contains a single copy of the luciferase sequence and each nuclear genome contains two copies of the actin sequence.

Luciferase assay

Luciferase assay was performed on cell lysates using the Promega (Madison, WI) Luciferase Assay System following the manufacturer's instructions. Briefly, 10 μl cell lysate was mixed with 100 μl substrate and luminescence was measured for 10 seconds in an MGM Instruments (Hamden, CT) Optocomp I luminometer. Lysates were diluted appropriately to allow for luminescence values within the linear range of the machine. Protein concentration was measured using the Bradford assay.

RESULTS

Isolation of nuclei with minimal extranuclear DNA

To prepare nuclei with minimal extranuclear plasmid DNA, we compared three nuclear isolation methods (Fig. 1a). For the first method ("detergent nuclei") [15], adherent cells were lysed with a detergent to remove plasma membranes, the remaining nuclei were scraped off of the surface, and the nuclei were separated from other cellular material by low-speed centrifugation. For the second method ("iodixanol nuclei") [13], adherent cells were trypsinized from the surface, lysed by dounce homogenization in a hypotonic buffer, and separated from other cellular material by high-speed centrifugation through an iodixanol density gradient. A third method that combined the first two was also tested ("combination nuclei"). For this method, nuclei scraped from a detergent lysed surface of adherent cells were

run through an iodixanol gradient in the same manner as for the iodixanol nuclei. Marker enzyme assays for cytosol (lactic dehydrogenase) and mitochondria (succinate dehydrogenase) indicated that nuclei isolated by each method did not contain detectable contamination from these organelles (not shown).

Twenty-four hours following transfection with polyethylenimine (PEI) or Lipofectamine (LFN), nuclei were isolated by one of the methods described above. The amount of plasmid DNA per nuclei was then determined by a relative quantitative PCR assay of total DNA extracted from isolated nuclei in which the amount of luciferase plasmid is normalized to a single-copy gene in the host genome, β -actin (Fig. 1b, see methods). Regardless of the transfection reagent, dose or cell type used, iodixanol nuclei consistently contained 10–100 fold lower plasmids/nucleus than detergent nuclei or combination nuclei. (Fig. 2a). To investigate whether this disparity is a result of a greater amount of DNA adhering to the outside of the nuclear membrane during the detergent isolation compared to the iodixanol isolation, we transfected B16F10 cells with fluorescently labeled polyplexes (Cy5-pLuc/Rhodamine-PEI) or fluorescently labeled lipoplexes (Cy5-pLuc/LFN) and isolated their nuclei by each method. Confocal microscopy images reveal that 75–95% of detergent nuclei are in nuclear aggregates (three or more nuclei) and that 100% of these aggregates contain a large amount of extranuclear polyplexes (Fig. 2b) or lipoplexes (not shown). On the contrary, no visible polyplexes (Fig. 2c) or lipoplexes (not shown) were seen on the outside of iodixanol nuclei, which are mostly dispersed (0–5% of nuclei in aggregates) (Fig. 2c). Nuclear clumping and the presence of extranuclear DNA were independent of the transfection method used (PEI or LFN). To test the feasibility of removing extranuclear DNA after nuclear isolation we next attempted to determine optimal nuclei wash conditions.

Testing conditions for removing extranuclear plasmid from isolated nuclei

To test the removal of plasmids from the outside of isolated nuclei, we prepared nuclei with luciferase plasmid present only on the exterior nuclear membrane by incubating luciferase plasmid with nuclei isolated from untransfected cells and evaluated various wash conditions for their ability to remove this extranuclear plasmid DNA (decrease the detectable plasmids/nucleus). Confocal microscope imaging of Cy5-plasmid-coated nuclei qualitatively confirmed the presence of plasmid on the exterior of the nuclei (Fig. 3a) and qPCR results showed that plasmid-coated nuclei had a similar amount of plasmids/nucleus as detergent isolated nuclei from transfected cells (not shown). Attempts to remove extranuclear plasmid from plasmid-coated nuclei by washing with greater than 100-fold excess of the cationic polymer spermine or anionic polymers polyaspartic acid and β gal plasmid, each of which could potentially compete with electrostatic interactions between the DNA and the nucleus, had limited or no success (Fig. 3b). Restriction digestion with MfeI, an enzyme that cuts within the luciferase sequence amplified in the qPCR assay, did however greatly decrease the detected plasmids/nucleus from the plasmid-coated nuclei (Fig. 3b). This result indicates that restriction digest of isolated nuclei could be used to render extranuclear DNA undetectable by PCR if it could be shown that the restriction enzyme cannot access intranuclear DNA.

To determine if enzyme digestion of nuclei is an efficient means of removing extranuclear plasmid without affecting intranuclear DNA, plasmid-coated nuclei were exposed to another restriction enzyme, MlyI, which cuts within both the plasmid luciferase (extranuclear) and the genomic actin (intranuclear) amplification sequences. As this experiment is designed to detect digestion of the genomic actin sequence, the actin sequence itself could not be used to normalize the amount of nuclei. Therefore a third gene, genomic gapdh, which is not cut by MlyI, was also included in the qPCR assay and the digestion of the luciferase and actin sequences was detected as a decrease in luciferase/gapdh and actin/gapdh, respectively.

When plasmid-coated nuclei are incubated with MlyI, 90–99% of the luciferase sequence is accessed and cut by the enzyme (Fig. 3c), as was the case with MfeI (Fig 3b). However, 35–40% of the actin sequence is also digested (Fig. 3d), likely through some perturbations in the nuclear membrane that occur during nuclear isolation. This indicates that the restriction enzyme is at least to some extent able to access intranuclear DNA. Furthermore, when nuclei are coated with polyplexes consisting of both plasmid and PEI, MlyI can again access and cut 35–40% of the genomic actin sequence (not shown) but can only cut 50% of the luciferase sequence (Fig. 3e), indicating that the PEI can protect the plasmid from digestion as has been previously reported [16]. When total DNA extracted from the polyplex-coated nuclei are incubated with MlyI, greater than 99% of the luciferase sequence is cut (Fig. 3e), indicating that the plasmid can be digested when freed from the polymer. However, if some or all of the DNA that arrives at the nuclear membrane during transfection is still compacted, a restriction enzyme will not be able to access all of the extranuclear DNA, further limiting the enzyme digest's usefulness.

A significant decrease in detectable plasmids/nucleus was also observed following enzyme digestion of detergent nuclei or combination nuclei from transfected cells (54 and 52% reduction, respectively), as indicated by the lower lines in Fig. 2a. However, the decrease in detectable plasmids/nucleus resulting from restriction digest of iodixanol nuclei from transfected cells was much less (17%) and not significant (again, lower line in Fig. 2a), further indicating that the iodixanol nuclei have minimal amounts of extranuclear plasmid.

Since the iodixanol method yields nuclei with less extranuclear DNA than is detectable by our fluorescent microscopy and enzyme digestion experiments without requiring additional washing steps, this method was used to prepare nuclei for all remaining experiments without any further washes following the iodixanol gradient separation.

Comparison of transfection vectors and cell types

To demonstrate the utility of the iodixanol nuclear isolation and qPCR method for determining the efficiency of plasmid delivery to the nucleus, we sought to compare the plasmid delivery efficiency of PEI and LFN at various doses. 80% confluent T-75 flasks ($\sim 10^7$ cells) of B16F10 or A549 cells were transfected with PEI polyplexes or LFN lipoplexes as described in materials and methods. 24 hours after transfection, nuclei were isolated from each flask of cells by the iodixanol method, total DNA was extracted from the isolated nuclei and this DNA was prepared for the qPCR assay to measure plasmids/nucleus (Fig. 4a,c). In addition, the amount of luciferase protein expressed by the transfected cells was determined by measuring the luminescence of the dounce homogenized cell lysates normalized to the amount of total cell protein (Fig. 4b,d).

Generally, nuclei isolated from transfected cells contained between 75 and 50000 plasmids per nucleus (between 1 and 5% of the applied dose) for both B16F10 cells and A549 cells (Fig. 4a,c). The remainder of the plasmid either does not enter the cell (only 10–20% of the applied dose is detected in total cell lysates, not shown) or the nucleus or gets degraded during the process of transfection. Surprisingly, nuclei isolated from LFN-transfected cells contained similar or slightly less plasmids/nucleus than nuclei isolated from PEI-transfected cells for each dose despite the fact that the LFN-transfected cells expressed 10–100 times more luciferase than PEI-transfected cells at these doses (Fig. 4). This result likely means that the plasmid DNA delivered to the nucleus by PEI is not as transcriptionally active as the plasmid DNA delivered by LFN and thus may be either still compacted with PEI or may be partially degraded. Therefore the most beneficial improvements to PEI as a DNA delivery system should be those that focus on improved protection of DNA and the release of the DNA from the polyplex.

Interestingly, even at doses at which luciferase expression is saturated (does not increase with a further increase in dose), plasmids/nucleus continues to rise. For example, the increase in B16F10 luciferase expression resulting from raising the dose of PEI polyplex from 2×10^5 plasmids/cell to 5×10^5 and 2×10^6 plasmids/cell is relatively small (1.3- and 1.4-fold, respectively) (Fig. 4b) while the corresponding increase in plasmids/nucleus is much larger (3.5- and 17.8-fold, respectively) (Fig. 4a). This is not a result of instrument saturation as all samples were diluted to ensure readings were within the linear range of luciferase and protein measurements. This result, also observed for LFN transfections in A459 cells (Fig. 4c,d), indicates that for each transfection reagent, conditions, and cell type there is an optimal dose of plasmid, above which any additional plasmid will not contribute to transgene expression even though it may be delivered to the nucleus. To improve the expression efficiency above this saturated level, one must improve the delivery system's ability to overcome barriers downstream from nuclear delivery, such as plasmid release (some of which may also occur prior to nuclear entry) and transcription/translation.

Plasmids/nucleus in cells sorted into high- and low-expressors

The plasmids/nucleus values reported thus far are an average over all of the cells in each transfection. However, we and others have observed by microscopic investigation (not shown) and cell-sorting of GFP-transfected cells (Fig. 5b,d) that there is significant heterogeneity of transgene expression within a population of transfected cells. To address this heterogeneity, we separated high expressing cells from low expressing cells within a single population of transfected cells and measured transgene expression and plasmids/nucleus for each subgroup.

B16F10 cells were co-transfected with 10^5 copies/cell each of a luciferase plasmid and a GFP plasmid and sorted for GFP expression after 24 hours (Fig. 5d). To confirm that the co-expression of luciferase does not affect sorting based on GFP fluorescence, B16F10 cells were also separately co-transfected with a secreted alkaline phosphatase plasmid (AP) and either the GFP plasmid or the luciferase plasmid. All three plasmids have a CMV promoter. A plot of FL4 (autofluorescence) vs FL1 (GFP fluorescence) for cells co-transfected with the luciferase plasmid and the AP plasmid (Fig. 5a) is indistinguishable from a similar plot for untransfected cells (Fig. 5c). Likewise, a plot of FL4 vs. FL1 for cells transfected with only the GFP plasmid (Fig. 5b) or cells co-transfected with the GFP plasmid and the AP plasmid (not shown) is indistinguishable from a similar plot for cells co-transfected with the GFP plasmid and the luciferase plasmid (Fig. 5d). We therefore conclude that luciferase co-expression in a cell does not affect its sorting based on GFP fluorescence.

B16F10 cells co-transfected with the luciferase plasmid and the GFP plasmid (Fig. 5d) were sorted for GFP expression after 24 hours into relatively high- (Fig. 5e) and low-expressing (Fig. 5f) cells. Luciferase expression (Fig. 5g) was correlated with plasmids/nucleus (Fig. 5h) for each group with high-expressing cells having almost 100x higher luciferase expression and greater than 3x higher plasmids/nucleus than low-expressing cells (Fig. 5i). In other words, cells that express higher amounts of transgene within a population of transfected cells also have a relatively high amount of plasmids in their nucleus.

DISCUSSION

To measure the amount of plasmids that reach the inside of isolated nuclei, it is important to ensure that the isolated nuclei are free from extranuclear DNA. Most previous studies [15, 17–20] have lysed cells with detergent and separated nuclei by slow speed centrifugation. We have found that nuclei isolated by this method are prone to aggregation and that these aggregates contain sufficient DNA outside of the nuclei to be detected easily by confocal microscopy (Fig. 2b). Furthermore, plasma-membrane lipid rafts have been detected in nuclei isolated from detergent-lysed cells [21]. Much effort has been made by us and other groups to

optimize wash conditions to remove extranuclear DNA from detergent-isolated nuclei which require cationic and anionic polymers and/or restriction endonucleases in addition to multiple centrifugation steps (Fig. 3, [15,18]). In this study we have investigated the use of mechanical lysing of cells followed by iodixanol density gradient ultracentrifugation [13]. This “iodixanol method” allows for the preparation of nuclei with minimal extranuclear DNA (Fig. 2c) without the need for additional wash and centrifugation steps that could disrupt the nuclei, although we cannot exclude the presence of a small amount of extranuclear DNA on the iodixanol nuclei that is not detectable by fluorescent microscopy or enzyme digestion. Nuclei isolated by a “combination method” in which cells were first lysed with detergent and then spun through the iodixanol gradient contained only slightly less plasmids/nucleus than nuclei isolated by the ‘detergent method’ (Fig. 2a). These results indicate that it is the detergent lysis step that causes extranuclear DNA to adhere tightly to the nuclei. Therefore the iodixanol method was used for further analysis.

To “count” the amount of unmodified DNA that is delivered to the nucleus, we have used a quantitative PCR assay [15,19,20]. In this paper, we extend this method with the use of the $2^{-\Delta\Delta C_t}$ relative quantification method [14] for accurately measuring the ratio of plasmids to a single-copy gene and thus the number of nuclei. This method has been used previously to detect the copy number of bacterial plasmids [22] as well as the copy number of chromosomal genes in mammalian cells [23,24]. Using real-time PCR to quantify plasmids in the nucleus provides a few important advantages: (1) a very large dynamic range of the amount of DNA over at least four orders of magnitude (Fig. 1b), (2) no need to modify the DNA with a label which could alter its intracellular trafficking, and (3) the ability to simultaneously quantify genomic DNA as a control for the number of nuclei in a sample. Using this method in conjunction with nuclei isolated by the iodixanol method described above, we are able to accurately detect the average amount of plasmids that are inside these nuclei and can use this information to gain insight into the intracellular events that occur during transfection.

Using these methods, we determined that nuclei isolated from B16F10 mouse melanoma cells or A549 human lung carcinoma cells transfected with LFN lipoplexes or PEI polyplexes contain between 75 and 3000 plasmids/nucleus, increasing with dose. Furthermore, increasing the number of plasmids per nucleus above 1000–3000 will lead to only marginal increases in expression (Fig. 4). Previous studies [15,20] have also observed a saturation of transgene expression with increasing plasmids per nucleus, emphasizing the importance of optimizing post-nuclear delivery events associated with transfection, including DNA release and transcription/translation. Although some studies using flow cytometry of isolated nuclei [18] or Southern Blot of DNA extracted from isolated nuclei [15,19] measure plasmids/nucleus in this same range, a recent investigation of nuclear delivery by LFN complexes in A549 cells by Hama et al [25,26] using fluorescently labeled plasmid DNA and a confocal microscopy technique measured greater than 4×10^4 plasmids/nucleus, more than 5 fold higher than what we measure with similar doses. This discrepancy can be accounted for by the fact that the confocal method is likely to overestimate the true amount of plasmids inside each nucleus because they are unable to distinguish between plasmids that are inside the nucleus and plasmids that are associated with the outside of the nucleus.

To demonstrate the utility of the methods described here for comparing transfection vectors, we compared the ability of PEI polyplexes and LFN lipoplexes to deliver plasmid DNA to the cell nucleus. At all doses tested, LFN and PEI delivered similar amounts of plasmids per nucleus (Fig. 4a, c) despite the fact that the LFN transfection resulting in significantly greater expression than PEI- typically 10-fold higher (Fig. 4b, d). These data indicate that the efficiency of expression from each plasmid delivered to the nucleus by PEI is less than that for plasmids delivered by LFN. One possible explanation is that PEI is still bound to plasmid in the nucleus to a higher degree than LFN is still bound to plasmid in the nucleus. Indeed it has been shown

that the release of DNA from transfection reagents is an important barrier to delivery [27]. Though it is not clear to what extent DNA release occurs before or after delivery of the DNA into the nucleus [12, 28, 29], considering proposed mechanisms of endosomal escape for each system could provide some insight into the likely fate of delivered complexes. With lipoplexes, cationic lipids interact with the anionic membrane lipids of the endosome leading to charge neutralization and therefore DNA release in addition to endosome destabilization [30]. In contrast, with PEI polyplexes, protonation of the polymer during endosome acidification leads to osmotic disruption of the endosome and release of the polyplexes into the cytoplasm without an associated release of DNA from the polymer itself [31, 32]. Therefore, according to these mechanisms, polyplexes would be more likely than lipoplexes to escape from the endosome and enter the nucleus intact, in agreement with our data. However, the exact mechanism of DNA release and nuclear entry is not known and it is possible that mechanisms that have thus far received little consideration are actually the most important for delivering the DNA to the nucleus that is ultimately transcribed. For example, DNA nanoparticles may bind to cell surface nucleolin and get shuttled directly from the plasma membrane to the nucleus in an endocytosis-independent manner [33] and lipoplexes in the cytoplasm may fuse directly with the nuclear membrane to release their contents to the interior of the nucleus [34]. Clearly, further investigation is necessary to fully understand the intracellular fate of gene vectors.

Without sorting transfected cells or focusing on individual transfected cells, any population measurement of plasmid delivery represents an average over all the cells in the population and does not provide information about sub-populations of cells with higher or lower than average expression. Transfection of cultured cells with nonviral vectors typically results in heterogeneous expression in which some cells in the same reaction vessel will express significantly more transgene than others. This phenomenon can be seen by sorting of GFP-transfected cells by fluorescence-activated cell sorting (FACS, Fig. 5a) or by simple observation of GFP transfected cells under a microscope (not shown).

Using cells transfected with fluorescently labeled plasmid encoding GFP, Tseng et al. [35] identified a correlation between uptake of plasmid into individual cells and GFP expression by those cells. In our cell sorting experiments, we were interested to know if there is also a correlation between transgene expression and uptake of plasmid into the nucleus. If nuclei from high-expressing cells contain a greater number of plasmids than nuclei from low-expressing cells then it would be reasonable to assume that the higher quantity of gene copies led to the greater amount of transgene expression. Alternatively, if nuclei from all cells contain the same amount of plasmids per nuclei then there must be some other characteristic about the high-expressing cells that allows them to have a higher rate of transcription of the delivered DNA.

To answer this question we co-transfected B16F10 cells with a GFP plasmid and a luciferase plasmid and then sorted the transfected cells by GFP fluorescence into high- and low-expressing cells (Fig. 5a). We then isolated the nuclei from each sub-population using the iodixanol method and quantified plasmids per nucleus in each using quantitative PCR (Fig. 5b). We found that indeed the high-expressing cells contain more than three times more plasmids per nucleus than low-expressing cells which corresponds to a two order of magnitude increase in luciferase expression (Fig. 5c). Thus, the high-expressing cells in a transfection reaction are the cells that took up the most plasmid into their nucleus. A similar 3-fold difference in plasmids per nucleus between high- and low-expressing has recently been shown in HEK293 cells [36]. It is likely that the high-expressing cells went through cell division during the duration of the experiment, allowing plasmid DNA to enter the nucleus during the breakdown and reformation of the nuclear membrane. This has been suggested previously as a major means of entry of plasmid into the nuclei of dividing cells [37,38] and is further supported by the fact that the high-GFP-expressing cells tend to be found in clusters that may have originated from the same parent cell (not shown). Furthermore, a recent paper by Mannisto et al [39] measured

greater than 10 times more plasmids in the nucleus of cells undergoing mitosis compared to cells in the growth phase of the cell cycle. Cells that have undergone mitosis may also have increased transgene expression because of decreased plasmid inactivation by cytoplasmic nucleases resulting from a shorter residence time of the plasmid DNA in the cytoplasm and because of a general increase in protein expression in the G1 phase of the cell cycle following mitosis [40]. Taken together, these results emphasize the heterogeneity of cells in a transfected population in regards to cellular uptake, nuclear uptake, and transgene expression, which is an important consideration if universal and/or uniform expression is desired (eg. expression of a suicide gene in tumor cells).

In this study, we have presented improved methodology for studying the extent of delivery of plasmid DNA into the nucleus of transfected cells. The nuclear isolation protocol we describe results in nuclei that contain far less extranuclear DNA than nuclei isolated with detergent without requiring additional washing steps. It is unclear if the minimum of 75 plasmids per transfected nucleus we have observed represents a minimum copy number necessary to overcome intracellular barriers. Indeed, Moriguchi et al [20] have shown that inclusion of dummy (non-coding) plasmid DNA with coding DNA in a transfection reaction can improve expression efficiency, presumably by swamping out some inhibitory event in the cells which would indicate that there is a threshold amount of DNA needed for expression. On the other hand, early microinjection studies by Mario Capecchi [41] showed that thymidine kinase (TK) activity could be detected in a TK-deficient mouse fibroblast cell line following the nuclear injection of as few as five TK-coding plasmids. For example, it has recently been shown that liver cells isolated from 6-week old Balb/c mice up to one week following hydrodynamic therapy with luciferase plasmid contain between 1 and 100 copies of the plasmid and exhibit detectable levels of luciferase expression [42]. These results raise the possibility that as few as one to five plasmids in the nucleus could be sufficient for low-level expression of the transgene.

The methods described here should be applied to additional gene transfer methods and reporter genes and to *in vivo* systems, in which the intracellular trafficking of plasmids could differ greatly from *in vitro* experiments [43]. Such studies will further enhance our understanding of the nuclear events that occur during transfection and could contribute to the design of novel vectors.

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Fig. 1. Nuclear isolation and quantitative PCR

(a) Diagram of the nuclear isolation methods used (see methods). (b) qPCR standard curves used for relative quantification of plasmids/nucleus. The threshold cycle (Ct) for various concentrations of plasmids containing either the luciferase sequence (pLuc), the genomic actin sequence (pAct) or the genomic gapdh sequence (pGap) were measured to calibrate the qPCR assay. The R^2 value and the slope of the fitted linear regression line and the calculated PCR efficiency ($E = 10^{(-1/\text{slope})} - 1$) for each plasmid are shown. The difference between the Ct of pLuc and the Ct of pAct ($\Delta C_{tL/A}$) over the same range of concentrations is also plotted (inset). Error bars representing the standard deviation of triplicates in the qPCR assay are smaller than the symbols representing each data point.

Fig. 2. Comparison of nuclear isolation methods

(a) Detected plasmids/nucleus using the PCR assay for nuclear DNA extracted from nuclei isolated from transfected cells using the indicated methods (Fig. 1a). *Indicates $P < 0.05$ (Student's t-test) in comparison to iodixanol nuclei from the same transfection conditions. For B16F10/PEI transfections, some isolated nuclei were treated with the restriction enzyme MfeI, which cuts within the luciferase sequence (but not actin or gapdh), before extraction of DNA and the resulting plasmids/nucleus values are indicated as the lower line in each bar. †Indicates $P < 0.05$ (Student's t-test) in comparison between digested and undigested nuclei. (b,c) Confocal microscope images of nuclei isolated from B16F10 cells transfected with rhodamine-PEI (red)/

Cy5-pLuc (blue) complexes (which appear pink) using either the detergent method (b) or the iodixanol method (c) and stained with Sybr I (green). Scale bar = 10 μ m.

Fig. 3. Testing wash conditions to remove extranuclear DNA from plasmid- and polyplex-coated nuclei

(a) Confocal microscope image of nuclei isolated from untransfected B16F10 cells by the detergent method, incubated with Cy5-pLuc for 1 hour at 4°C and washed with lysis buffer. Scale bar = 10 μm. (b-e) Nuclei were coated with pLuc or PEI/pLuc polyplexes as in (a) but with non-fluorescent materials. (b) Detected plasmids/nucleus for pLuc plasmid-coated nuclei following the indicated nuclear washing steps. (c) Detected relative ratio of luciferase plasmid DNA to gapdh genomic DNA for pLuc plasmid-coated nuclei exposed to MlyI before or after DNA extraction. (d) Detected relative ratio of actin genomic DNA to gapdh genomic DNA for pLuc plasmid-coated nuclei exposed to MlyI before or after DNA extraction. (e) Detected

relative ratio of luciferase plasmid DNA to gapdh genomic DNA for PEI/pLuc polyplex-coated nuclei exposed to MlyI before or after DNA extraction. Error bars represent standard deviation of triplicates in the qPCR assay.

Fig. 4. Plasmids/nucleus and luciferase expression dose response of PEI polyplexes and LFN lipoplexes

B16F10 (a–b) or A549 (c–d) cells were transfected with PEI/pLuc polyplexes or LFN/pLuc lipoplexes at the indicated doses. (a,c) Detected plasmids/nucleus in nuclei isolated by the iodixanol method. (b,d) Detected luciferase expression in lysates from transfected cells using the Promega Luciferase Assay kit normalized to the amount of total protein determined by the Bradford assay. Error bars represent the standard deviation of triplicates in the qPCR assay or luciferase assay.

Fig. 5. Measuring plasmids/nucleus in subpopulations of high- and low-expressing cells
B16F10 cells were co-transfected with 10^5 copies/cell each of PEI/pLuc and PEI/pAP (secreted alkaline phosphatase) polyplexes (a), PEI/pGFP only (b), no polyplexes (c), or PEI/pLuc and PEI/pGFP polyplexes (d). Trypsinized cells were then analyzed for GFP expression using a MoFlo fluorescence-activated cell sorting (FACS) machine. The GFP intensity was measured in the FL1 channel with the FL4 channel used as an internal autofluorescence control. Forward- and side-scatter analyses (not shown) were used to exclude cellular debris contamination of sorted cells. Cells co-transfected with PEI/pLuc and PEI/pGFP polyplexes (d) were sorted into groups of relatively low (e) and high GFP expression (f). Luciferase expression (g) and plasmids/nucleus (h) were then measured for each group. *Indicates $P < 0.05$ (Student's t-test)

in comparison to transfected-unsorted cells. In (i) the data from (g) and (h) are re-plotted against each other. Error bars represent the standard deviation of triplicates in the qPCR assay or luciferase assay.