A simple and rapid nonviral approach to efficiently transfect primary tissue–derived cells using polyethylenimine

Charlie Yu Ming Hsu[1] & Hasan Uludağ[1–3]

[1]Department of Biomedical Engineering, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada. [2]Department of Chemical and Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada. [3]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada. Correspondence should be addressed to H.U. (hasan.uludag@ualberta.ca).

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This protocol outlines steps for optimizing the transfection of adherent primary mammalian cells using the readily available off-the-shelf cationic polymer, 25-kDa branched polyethylenimine (bPEI25). Transfection efficiency of cationic polymers varies among cell lines and is highly dependent on the conditions and environment in which complexes are formed. Factors requiring optimization include the salt concentration, volume, incubation time, mixing order and ratio of polymer to DNA. In this transfection protocol, complexes are prepared in 30 min, with analysis 24 h later; thus, experiments can be completed in 2 d. In this protocol, as an example, we describe the parameters we have optimized for the transfection of bone marrow stromal cells and normal human foreskin fibroblasts. By using this protocol, we have obtained transfection efficiencies comparable to lipofection. An appropriately optimized protocol enhances the utility of cationic polymers in transfecting mammalian cells, thereby providing an effective alternative to expensive commercial reagents.

INTRODUCTION

Exogenous nucleic acid molecules can be artificially introduced into mammalian cells using viral vectors, physical methods or biocompatible cationic materials. Viral vectors are the most efficient at transducing cells, owing to their naturally evolved mechanism to evade cellular barriers. However, immunogenic components of viral vectors may limit their utility in sensitive applications. In addition, the construction and packaging of viral vectors can be technically demanding and require specialized lab equipment. Physical methods of delivery such as electroporation and microinjection provide safer alternatives to viral vectors, but the transfection window and efficiency are typically narrow and low, and the requirement for specialized equipment and devices can be expensive and limit accessibility. Nonspecific damage to the cells is a further concern that limits the utility of physical transfection methods. Biocompatible cationic polymers and lipids have a wide transfection range and relatively low immunogenicity. They are readily available as off-the-shelf reagents; carrier-DNA complexes are formed by self-assembly through electrostatic interaction between the cationic reagents and the anionic nucleic acid. The complexes can then be added directly to the growth medium, without a need for specialized devices. Thus, nonviral gene carriers provide a more accessible option for routine genetic manipulation of cellular physiology in studies ranging from gene function to *ex vivo* therapeutics.

Cationic polymers

Among the first cationic polymers to be used in transfection was polyethylenimine (PEI). PEI comes in linear and branched configurations, ranging in molecular weight from 800 to 1,000,000 Da (ref. 1). Its ability to transfect is principally derived from the high density of positive charges attributed to amine groups, which interact electrostatically with the negative charged phosphate backbone of nucleic acid to condense both molecules into submicrometer particles that can bind to the cell surface and be taken up via endocytosis. The abundance of amine groups further provides a 'proton-sponge' effect in which absorption of protons (H^+) by the amine groups inside the endosome leads to osmotic swelling and eventual rupture of the endosome membrane to release the endocytosed cargo into the cytoplasm[2]. Transfection efficiency of PEI is closely tied to nucleic acid binding and dissociation of the polymer as it relates to the packaging and release of nucleic acid cargo. Low-molecular-weight PEIs have fewer amine groups per molecule, which bind and condense DNA less efficiently, translating into lower overall transfection efficiencies. High-molecular-weight PEIs have stronger binding affinity, which can condense DNA more efficiently, but they have a less effective release, leading to reduced transfection efficiency. High-molecular-weight PEIs are also more toxic, reducing the viability of cells for transgene expression. Therefore, mid-range–molecular-weight PEIs provide a balance between binding affinity and ease of dissociation. As such, bPEI25 and 22 kDa linear PEI are the most popular and most effective polymeric transfection agent cited to date[3].

Despite the ability of PEI to transfect a wide range of cell types, achieving a transfection efficiency suitable for the desired application remains a challenge. Although the development of modified polymers is an ongoing area of research, the methods in which complexes are formed is often overlooked. The transfection utility of PEI polymers is highly dependent on the environment and conditions in which complexes are formed. In some cases, complexes formulated under one condition for transfecting a particular cell line may not necessarily be optimal for another cell line. Thus, the transfection protocol should be specifically optimized for each polymer and each type of cell line. This is crucial to ensure that the most utility can be derived from the transfection reagent as well as to ensure that the comparative evaluation of transfection efficiencies between existing polymers and novel gene carriers is achieved.

Experimental design

The overall procedure for transfection is outlined in **Figure 1** and can be envisioned as having two main parts. The first part involves the mixing of DNA and polymer in a smaller volume

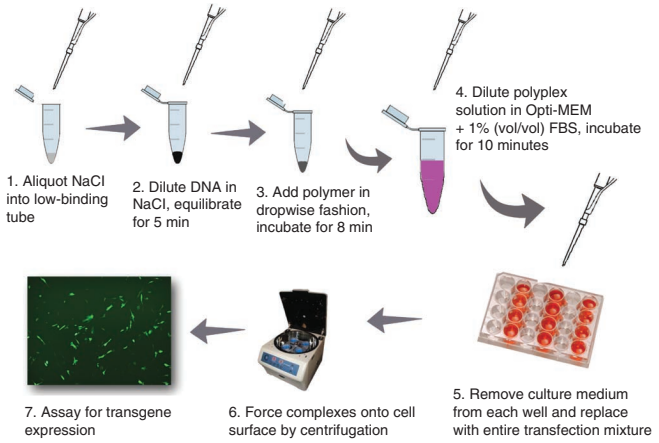

Figure 1 | Schematic overview of the suggested transfection protocol.

of aqueous solution followed by minutes of incubation to allow complexes to mature. The second part involves the addition of complexes to the cell in a larger volume of growth medium and incubation for a few hours to allow adsorption and uptake of complexes. A number of parameters can affect the physicochemical properties of the complexes in both parts of the transfection process, including salt concentration, types of solute, volume of solution, pH, incubation time, viscosity, buffers, concentration of DNA, amount of polymer, polymer-to-DNA ratio, mixing order, mixing speed, temperature, presence of amphiphatic molecules (e.g., surfactants) and ion species. As transfection is typically done in minimum essential growth medium, the condition in which complexes are formed needs to be compatible with the growth medium to preserve cell viability. As such, we focus our discussion on optimizing parameters that can be practically implemented under physiological conditions.

Cell line. Transfection efficiencies are known to vary greatly among mammalian cell lines. This is principally due to differences in cell physiology, which dictates metabolic requirement, and can affect the distribution of cell surface receptors and the uptake pathways used by the cell to internalize complexes[4,5]. Further, immortalized cultured cells such as COS-7, NIH/3T3, HeLa, HEK 293T and CHO cells can be transfected much more readily to higher efficiency than tissue-derived primary cells such as fibroblasts and bone marrow stromal cells (BMSCs)[6,7]. The process of transforming cells to make them more amenable to culture conditions may have inadvertently altered some parts of the cellular processes, such as cell cycle and uptake pathways that made them more susceptible to transfection. In contrast, primary tissue–derived cells could be more selective toward the physicochemical properties of the complexes, which can define the predominant endocytic uptake pathway and ultimately how cargos are processed and transported within the intracellular domains. Thus, it is important to note that transfection procedures need to be optimized for individual cell line.

Complexation volume. Studies often report the amount of DNA used in transfection as a concentration of the final medium volume after complexes are added to the cell. This concentration is not the same as the concentration of DNA during complexation, which can drastically affect how complexes are formed. When complexes are formed at high concentrations of DNA/polymer, the increased frequency of intermolecular interactions may not provide sufficient spacing to allow proper DNA condensation and maturation of individual particles, which can lead to partially formed particles and aggregation—features that are less desirable for efficient transfection. Therefore, dilution of DNA and polymers in a larger volume generally leads to smaller and more uniform particles that are more conducive to transfection. However, if the volume of the complexes becomes too large, it might dilute essential nutrients required to sustain metabolic activity in the growth medium after the addition of complexes. Thus, optimal volume for complexation will need to be empirically determined as a function of DNA concentration, nitrogen-to-phosphate (N/P) ratio and growth medium volume.

Salt and solutes. The effect of solutes on particle stability varies with the structure and molecular weight of the PEIs. For example, 22 kDa linear PEI (LPEI22) and bPEI25 can both form complexes <100 nm in size under salt-free conditions. However, when physiological salts were added to the complexes, LPEI22 complexes grew into large aggregates, whereas bPEI25 complexes remained small[3,5]. This suggests that complexes may undergo physicochemical changes when mixed with media of different solute concentrations. That is, when complexes are added to the growth medium for transfection, the sizes and stability of the particles may change during mixing of the two solutions. Regardless of what the sizes of the complexes formed in salt-free or saline solution are, both should be tested as part of the optimization experiment to determine the best condition for transfection. In our experience, we have found that bPEI25 complexes formed in salt-free solution (20 mM HEPES, pH 7.4) lead to considerably higher transfection than complexes formed in buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.4). However, the opposite is true for transfection in BMSCs, in which complexes prepared in buffered saline lead to better transfection. Thus, a universal complexation method may not yield optimal transfection protocol in every cell line.

DNA concentration, ratio of polymer-to-DNA and polymer concentration. In general, the higher the concentration of DNA administered to the cells, the higher the level of transfection. However, the amount of DNA that can be applied in transfection is limited by the final concentration of polymer and the ratio of polymer to DNA. The optimal polymer-to-DNA ratio (or nitrogen-to-phosphate ratio) is often in excess of the ratio at which full binding and full condensation occurs. This means that the solution of complexes often contains an excess of unbound polymers as well. PEI chains bound to DNA mainly condense and protect the cargo, but free PEI appears to be essential for intracellular trafficking and for overcoming the inhibitory effect of the anionic cell-surface glycosaminoglycan (GAG)[8–10]. However, if the amount of PEI becomes too high, cell damage may ensue and reduce overall viability. Thus, optimal transfection conditions would occur at a polymer amount just sufficient to overcome the inhibitory effect of GAG while providing robust complex uptake.

Optimizing the three parameters involves first determining the upper limit of polymer concentration the cells can withstand, then complexing with various amounts of DNA at the upper polymer concentrations to test in transfection. The polymer-to-DNA ratio that gives the highest level of transfection effectively determines the upper limit for DNA concentration. As cell physiology can affect a range of metabolic activities, including uptake pathway and the expression of cell surface receptors, the amount of surface GAG is likely to differ between cell lines. Thus, the optimal polymer concentration and polymer-to–DNA weight ratio need to be empirically optimized for each cell line.

Effect of serum. Serum protein in growth medium can interact with complexes to form large aggregates that reduce and inhibit transfection efficiency. The effect of serum on transfection varies between cell lines[11]. For example, HEK293T cells can be transfected in growth medium supplemented with 10% (vol/vol) FBS without a marked reduction in reporter gene expression. In contrast, transfection of primary tissue–derived cells such as fibroblast and BMSCs in the presence of serum nearly abolished reporter gene expression[12]. The amount of serum in the growth medium during transfection will require optimization with respect to the polymer and the cell line. In our experience, the presence of any serum in the transfection medium of fibroblasts substantially reduces transfection efficiency. However, low amounts of serum (e.g., 1% (vol/vol) FBS) for the transfection of bone marrow cells leads to better transfection in comparison with complete serum-free medium. As serum is required for metabolic activity and cell viability, the sensitivity of the cells to the absence of serum during the transfection incubation time may vary from cell line to cell line.

Complex stability and incubation time. Once complexes are formed, the utility time frame for transfection is limited—complexes are unstable in the solution and will gradually form large aggregates over time. Aggregation can arise because of heterologous intermolecular interaction with serum protein through charge-charge interaction, and/or through homologous interaction with other PEI complexes as a result of hydrophobic shielding[13,14]. Although the overall charge of the complex is positive, particles can exist as amphiphatic molecules with pockets of hydrophobic regions[14]; thus, particles may spontaneously bind to each other to shield these hydrophobic pockets from the aqueous solution, forming aggregates. Large aggregates are less efficiently taken up by the cell, are not as readily dissociated and can lead to increased toxicity, resulting in dramatic reduction in transfection efficiency. Because of this time sensitivity, complexes are typically incubated with cells for a limited time frame (i.e., less than 24 h, typically between 2 and 24 h). However, one of the limiting factors in transfection is the diffusion barrier in the liquid medium where particles need to sediment down by gravity to the bottom of the plate in order to bind to the cell surface. The time delay for this process ranges from 2 to 6 h. Because of the time-sensitive nature of the particles' stability, the diffusion barrier may effectively limit transfection efficiency. Methods to overcome this barrier include centrifugation to force the particle onto cell surface or magnetofection, in which magnetized PEI complexes are pulled down by a magnetic field[15].

Cell density. The density of cells during transfection is closely tied to the polymer and DNA concentrations. If cell density is low, the concentration of polymer would be relatively high compared with transfection in a densely populated cell culture. Many transfection protocols cite a certain number of cells per well as the seeding density. However, it is important to keep in mind that seeding density does not necessarily translate to attached cell density; attachment efficiency can vary from batch to batch, depending on culturing conditions, handling processes and age of culture. Thus, in a protocol in which cell seeding is recommended 24 h before transfection, it is more crucial to check that the cells have reached the desired density for transfection rather than to follow a set time frame for the experiment.

Culturing condition. Cell physiology greatly influences transfection efficiency in carrier-assisted gene delivery[6,7,16]. Although cell types remain an unchangeable factor in an experimental setup, their metabolic activity and growth rate can be maximized to enhance transfection. Transfection efficiency is directly correlated to cell cycle; both the S-phase and the M-phase contribute to enhanced transgene expression as a result of elevated global transcriptional activity during DNA synthesis and plasmid DNA (pDNA) nuclear import during transient disassembly of the nuclear envelope[17–19]. Thus, cells should be maintained in a highly active dividing mode to passively enhance transfection efficiency.

Cultures that are grown past the confluent stage generally start to show lower metabolic activity; this slowdown of the growth rate can be passed down to subsequent generations and may require a few additional passages before the growth rate can resume. We advise subculturing cells when the density reaches 80% or every 5–7 d to maintain cells in an actively dividing mode. However, cells with high passage numbers are generally less metabolically active and will eventually go into senescence. Thus, if experiments are not to be performed immediately, freeze the cells in a cryogenic vial and store them at −80 °C until needed.

Seeding density can also influence growth rate. As adherent cells require attachment and presence of cells to some extent for growth (without excessive cell-to-cell contact that impede growth), cells grown at a low starting density (e.g., 20–30%) will grow slower than a culture with a higher starting density (50–60%) and will reach the desired transfection density at different rates.

Contamination with mycoplasma can also pose severe challenges to transfection. Periodically monitor cultures for infection using a mycoplasma detection kit. If infection is found, either discard the culture or remove the intracellular microbe using a cleanup kit.

Choice of promoter and reporter gene construct. Regulatory and genetic elements on the pDNA vector can have a major effect on the level of transgene expression. It is widely observed that promoter activity can vary from cell line to cell line with respect to the amount of protein expressed and the duration of expression[20–24]. Constitutive promoters derived from viruses such as cytomegalovirus (CMV) and Rous sarcoma virus (RSV) are most commonly used for high-level expression. However, viral promoters are subjected to epigenetic silencing over time and thus ubiquitous promoters from nonviral sources such as the human elongation factor 1-α (EF1-α), human polyubiquitin C and chicken β-actin[20,22,25] are common alternatives. In our experience, the gWIZ series of vectors (Aldevron), which contain a recombinant CMV IE/intron A promoter, were substantially more efficient at transfecting cells than the first generation of CMV-based vectors, such as the pEGFP-N2. Because promoter strength and activity is highly dependent on cell type, we recommend testing a series of expression pDNAs under the control of different promoters, in order to optimize the level and duration of transfection that is ideal for your application.

Aside from positive regulatory elements, noncoding sequences found on the pDNA vector can have an inhibitory effect on overall transfection efficiency. Bacterially-derived vector backbone can induce strong innate immune response and lead to the production of proinflammatory cytokines[26–28]. Further, high abundance of unmethyled CpG dinucleotides that are characteristic of the bacterial sequences can induce heterochromatization, rendering pDNA in a transcriptionally inactive state, and reducing both the level and duration of transgene expression[27–29]. As such, pDNAs devoid of vector backbone, commonly termed minicircle DNA, have been shown to have enhanced transgene expression and transgene persistence[30–32]. Replacement of the expression construct with a CpG-depleted minicircle pDNA may be an option for providing enhanced transgene expression.

Limitations of the protocol. Although this protocol optimizes the utility of off-the-shelf cationic polymers, the inherent limitations lie with the polymers and the cell lines. Transfection efficiency is highly dependent on the sizes of the polyplex particles. Polymers with lower binding affinity (e.g., 2 kDa PEI) tended to form less condensed and charged (ζ-potential) complexes, which result in larger particles that are less efficiently taken up in comparison with polymers that can condense pDNA into a more stable and compacted structure (e.g., bPEI25). Cell physiology dictates endocytic uptake pathway, growth rate and sensitivity to the polymer, which are crucial factors in determining transfection outcome. That is, cells that take up complexes predominantly through a nonendosomolytic pathway (e.g., CHO and COS7 cell[7]) limit the capacity for PEI complexes to use the proton sponge effect to escape the endosome. In addition, transfection efficiency is correlated with cell division, and slowly dividing cells may limit nuclear import of pDNA for subsequent transgene expression. Further, sensitive cells impose a lower limit on the concentration of the polymer, which equates to lower concentration of pDNA, essentially limiting transfection efficiency.

Another inherent limitation is the duration of transfection, which is expected to be transient. Maximum transgene expression is expected to be between 1 and 3 d after complex addition but will gradually decline thereafter and only remain detectable up to 7 d. A mechanism for replicating and partitioning the pDNA throughout cell division is required to maintain a minimum intracellular copy number for transcription. Additional genetic elements are also needed for nuclear retention and to maintain the transgene in an open chromatin state for long-term expression. Typical mammalian expression constructs lack these epigenetic elements for transgene persistence and thus can only provide transient transfection.

It is important to note that although this protocol may provide enhanced transfection utility of bPEI25, there remains a disparity between desired level of transfection and optimal transfection efficiency of the polymer. The desired level of transfection is highly dependent on the types of protein being expressed and the minimum concentration for bioactivity, and may not be achievable, despite optimal conditions. The development of novel biomaterials for gene delivery is an ongoing research area aimed to address this issue and is beyond the scope of this protocol.

Optimization required for NHFFs and rat bone marrow and human stromal cells. In this procedure, we provide a step-by-step protocol optimized for the transfection of normal human foreskin fibroblasts (NHFFs, CRL2522), rat BMSCs and human stromal cells using bPEI25 (ref. 33). We note that a similar protocol is available for the transfection of epithelial cells[34]. The major difference between these two protocols lies in the preparation of complexes and the transfection conditions. We found that complexes formed by direct mixing in buffered saline work well in most cases, whereas in some cells others have found mixing of two equal volumes of DNA and polymer solution in HEPES to be optimal[31]. We believe that both methods work well, but the optimal method will depend on the cell lines. Transfection in a 24-well or larger format is recommended when you have the intention of adapting transfection for protein expression and *ex vivo* application, whereas a 96-well format may be better suited for high-throughput screening. For transfection, we include a centrifugation step with incubation in the presence of reduced-serum medium, Opti-MEM (instead of DMEM), in order to facilitate cell binding of complexes in a shorter time and to better preserve cell viability in the absence of serum.

Although this protocol is written for transfection with pDNA for transgene expression, many of the concepts discussed so far (e.g., volume, buffer, solutes and ratios) can be applied to siRNA delivery as well. This protocol might be beneficial to researchers who are developing novel biomaterials for gene delivery as guides to ensure that carriers are evaluated at the optimal efficiency. Researchers requiring transfection reagents for routine biological studies might also find this guide useful in updating their transfection procedures. This protocol may also offer an alternative to commercial reagents such as Lipofectamine 2000, especially considering that bPEI25 is a cost-effective reagent (>500 times cheaper per 'transfection') in comparison with specialty reagents.

MATERIALS

REAGENTS

- Cell line of interest (we use NHFF (CRL2522), rat BMSCs and human stromal cells)
- Basic cell culture growth medium: DMEM (low glucose) with L-glutamine and sodium pyruvate (see REAGENT SETUP)
- Fetal bovine serum (FBS; Gibco, cat. no. 12483-020)
- Penicillin-streptomycin solution (liquid: 10,000 U ml^{-1} penicillin/10,000 μg ml^{-1} streptomycin; Invitrogen, cat. no. 15140-122)
- Trypsin-EDTA solution (1× liquid, 0.05% (wt/vol) trypsin/0.53 mM EDTA)
- PEI (branched, 25 kDa; Sigma-Aldrich, cat. no. 408727)
- OPTI-MEM reduced serum medium (Invitrogen, cat. no. 31985-070)
- HEPES buffered saline (HBS, 150 mM NaCl; see REAGENT SETUP)
- CMF-Hank's balanced salt solution (CMF-HBSS), without Ca^{2+} and Mg^{2+} and with phenol red
- Mammalian expression plasmid containing the reporter gene *GFP* under the control of the hybrid CMV IE/Intron A promoter (Aldevron, cat. no. 5006)
- Sodium chloride (NaCl)
- HEPES
- ddH_2O
- pDNA
- Formalin

EQUIPMENT

- Stericup filtration system (0.22 μm; Millipore)
- Inverted phase contrast microscope
- 5% CO_2 water-jacketed incubator
- Tissue culture flask (75 cm^2) with gas exchange ventilation cap
- 24-well flat bottom tissue culture treated plates
- Water bath (37 °C)
- Low-binding polypropylene microcentrifuge tubes
- Centrifuge tube (50-ml) with conical bottom (sterile)
- Multipurpose centrifuge with swing bucket rotor and microplate adaptor rotors (e.g., Eppendorf 5810 or similar with A-4-62 and A-2-DWP rotors)
- Pasteur pipettes
- Flow cytometer with argon laser (488 nm)

REAGENT SETUP

Cell culture medium DMEM with 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin supplemented with 10% (vol/vol) FBS (heat-inactivate FBS at 56 °C for 30 min before adding it to DMEM). Growth medium should be stored in a 4 °C refrigerator for up to 4 weeks.

HEPES buffer (20 mM HEPES, pH 7.4) Make up 1 M stock solution by dissolving 2.383 g of HEPES (*N*′-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid) in 100 ml of ddH_2O; adjust to pH 7.4 with KOH. Mix 1 part of 1 M

HEPES to 49 parts of ddH_2O to make the 20 mM HEPES solution. Solutions can be made in advance and stored at room temperature (i.e., 21–23 °C) for up to 1 year.

HBS Dissolve 0.8766 g of 150 mM NaCl in 100 ml of 20 mM HEPES (pH 7.4); pass it through a 0.22-μm filter to sterilize and remove any particulates. Solutions can be made in advance and stored at room temperature for up to 1 year.

Plasmid DNA solution Dilute in RNase-free, DNase-free water to a final concentration of 0.4 mg ml^{-1}. DNA solution can be stored in a 4 °C refrigerator for up to 1 week or in a 20 °C freezer for long-term storage.

bPEI25 solution Dissolve 10 mg of bPEI25 in 10 ml of deionized RNase-free, DNase-free water. All PEI polymers are sticky, viscous sap-like resins; when weighing the polymer, dispense it directly into a low-binding polypropylene tube to ensure that an accurate amount is weighed. Vortex it rigorously and allow the solution to sit at room temperature for at least 24 h before use to ensure complete dissolution. Store at 4 °C for up to 5 years.

PROCEDURE

Revive frozen cell stock for subculturing ● TIMING 10 min for seeding (5–7 d before subculturing for transfection)

1| Cover an empty sterile 75-cm^2 flask with 10 ml of prewarmed cell culture medium. Roll the flask gently to ensure even coverage of the bottom surface.

2| Remove the frozen cell stock from −80 °C freezer and thaw the cells in 37 °C water bath. For the procedure for isolating tissue-derived fibroblasts and BMSCs, please refer to refs. 35–37.

3| Check the vial every minute; once the vial has thawed, immediately aliquot approximately 5 × 10^6 cells into the tissue culture flask.

▲ CRITICAL STEP The number of cells seeded into each flask is provided as a guideline, as the actual number of cells attached to the surface will vary and depend on the storage, handling conditions, cell freezing medium, cell line, batch and passage number. Generally, an initial seeding density with 30–40% attachment should take about 5–7 d to reach 80–90% confluence.

4| Place the flask in a 37 °C incubator and allow the cells to attach to surface. After 4 h, change the medium.

? TROUBLESHOOTING

5| Return the flask to the incubator and allow 5–7 d for cells to become confluent. Check cells under the microscope daily to ensure that there is an increase in density. Once cells have reached 80% density, proceed to Step 6.

▲ CRITICAL STEP Cells will remain at the tail end of the log-phase at 80–90% confluence. Do not allow cells to grow for longer than 2 weeks, as cells will start to die off and/or become senescent with reduced metabolic activity. This could substantially affect transfection efficiency in subsequent passages. Once cell density increases beyond 80%, fibroblasts begin to show an elongated compacted morphology; this is an indication that they are no longer in the exponential growth phase.

Cell seeding for transfection (24-well plate) ● TIMING 30–40 min

6| Once cells are 80–90% confluent, aspirate the cell culture medium with a sterile Pasteur pipette, and then wash the cells twice with prewarmed CMF-HBSS (Ca^{2+}-free and Mg^{2+}-free HBSS with phenol red) for 5 min per wash.

▲ CRITICAL STEP Check to ensure that the HBSS does not contain calcium and magnesium. The presence of the cations during the washes will prevent cells from detaching. Two or more washes may be necessary to sufficiently detach the cells. The first wash is to dilute and remove residual serum and traces of divalent cations from the surface; subsequent washes are to equilibrate the cells with the wash buffer.

7| Aspirate CMF-HBSS and detach the cells by adding 5 ml of 1× liquid 0.05% (wt/vol) trypsin/0.53 mM EDTA, and then swirl to ensure even coverage across the flask surface. Leave at room temperature for 2 min.

8| After about 2 min of contact with trypsin, gently tap the sides of the flask to agitate and loosen cells from the surface.

▲ CRITICAL STEP Allow sufficient time for trypsin to equilibrate before tapping. Excess agitation may cause cells to aggregate, leading to patches of cells in each well after seeding. The ease at which cells can be detached with trypsin is dependent on the adhesion property of the cells and their sensitivity to trypsin, which vary among cell types. Although detachment with trypsin can be enhanced by incubation at 37 °C, extending the incubation time (2–10 min) or using a more concentrated trypsin (0.25% (wt/vol)) may also increase the risk of cell damage. Detachment with a cell scraper or a cell lifter is NOT recommended.

? TROUBLESHOOTING

9| Once cells have detached, stop trypsin activity by adding cell culture medium (FBS contains a trypsin inhibitor).

10| Transfer the cell suspension into a 50-ml conical tube and pellet the cells by centrifugation at 600 r.p.m. (72*g*) for 5 min at room temperature.

11| After centrifugation, a cell pellet should be visible at the bottom of the tube. Aspirate the supernatant, being careful not to disturb the cell pellet.

12| Suspend cells in 48 ml of cell culture medium (1:4 split).
▲ **CRITICAL STEP** The resuspension volume will depend on the split ratio or the desired seeding density. Typically, an 80% confluent flask is split 1:4 to obtain the ideal density of 40–50% confluence per well for transfection. If the cell suspension requires dilution beyond this split ratio, it may be an indication that cells are overgrown.

13| Aliquot 500 µl of the suspended cells or approximately 60–70 × 10^3 cells into each well of the 24-well plate.

14| Gently shake the plate to ensure that cells are uniformly distributed throughout the well surface.
? TROUBLESHOOTING

15| Place the seeded plates back in the incubator. Check the plates under the microscope every 5 min to ensure that cells are evenly distributed across the well.
▲ **CRITICAL STEP** Before attachment, cells tend to aggregate toward the center of the well. Visualize the cells under a phase-contrast microscope at low magnification (×2.5) and ensure that cells are evenly distributed by gently shaking the plate. Cells will begin to attach once the plate temperature has equilibrated back to 37 °C (or about 10 min after the plate is placed back in the incubator), so check the plate for aggregation every 5 min prior. If they are not distributed evenly, cells concentrate at higher density in the center, thereby creating a topographical density gradient that can substantially affect reproducibility.

16| Check the cells under a microscope after 24 h; if the cells are at 40–50% density, proceed to transfection. If not, wait an additional 1–2 d before transfection.
▲ **CRITICAL STEP** We emphasize the splitting ratio as a starting cell density over a particular number of cells, as the latter does not always correlate to the number of cells attached to the surface. Attachment efficiency is highly dependent on the growth rate, passage number and handling during the trypsin stage.
? TROUBLESHOOTING

Preparation of bPEI25/pDNA polyplexes for transfection ● TIMING 30 min
17| Make transfection complexes. Transfection efficiency among cell lines may be dependent on complexation methods. We provide two methods below: direct mixing in buffered saline (option A) and two-part mixing in salt-free buffer (option B). Both may need to be tested to determine the optimal condition. The following volumes and concentrations have been described for transfection per well in a 24-well plate. Maintain the same relative proportions when dispensing in replicates. Adjust the volume accordingly for 12- and six-well plates (**Table 1**). Transfection in 48-well plates is not recommended, as the small volume generally results in unstable aggregates that can lead to sporadic toxicity.
▲ **CRITICAL STEP** The volume and incubation time listed in both options A and B has been optimized for the concentrations of pDNA and polymer. If cells can withstand a higher concentration of polymer, then both the incubation time and volume may need to be adjusted proportionally to ensure stable complexes are formed.
▲ **CRITICAL STEP** We recommend transfecting in the absence of any antibiotics. Cell viability and membrane integrity may be compromised during interaction with polyplex and polymer, which may cause antibiotics to leak into the cell. Although data on this for polyfection are limited, antibiotics have been shown to reduce transfection and increase cell death in lipofection[38].

TABLE 1 | Volume of individual components in transfection solution per well (µl).

Components	48-well	24-well	12-well	6-well
150 mM NaCl	Not recommended	45	90	180
pDNA (0.4 mg ml^{-1})		2.5	5	10
bPEI (1 mg ml^{-1})		2.5	5	10
Opti-MEM (1% (vol/vol) FBS)		450	900	1,800

(A) Direct mixing with buffered saline

(i) In 45 µl of buffered saline (150 mM NaCl, 20 mM HEPES (pH 7.4)) add 2.5 µl of pDNA (0.4 mg ml^{-1}); mix and allow the pDNA to equilibrate for 5 min at room temperature. (The final pDNA concentration per well in this setup is 2 µg ml^{-1}.)

▲ **CRITICAL STEP** bPEI25 may precipitate out of solution during cold storage; allow all components to equilibrate to room temperature before proceeding with the preparation of complexes.

▲ **CRITICAL STEP** bPEI25 may bind to tube side walls[39], effectively lowering the concentration of the polymer upon prolonged storage; be sure to use low-binding polypropylene tubes in all steps.

(ii) Add 2.5 µl of bPEI25 (1 mg ml^{-1}) to the diluted DNA solution slowly, in a dropwise manner. The polymer-to-DNA weight ratio is 2.5, and the final polymer concentration per well is 5 µg ml^{-1}.

▲ **CRITICAL STEP** It is crucial that the sequence of addition is followed exactly as described (i.e., aliquot the buffered solution, add DNA to the solution and then add PEI to mix). The volume of the initial polyplex solution should be at least one-tenth of the final transfection solution.

(iii) Mix the solution by vortexing for 5 s, and then allow the solution to sit at room temperature for 10 min.

(iv) Dilute the polyplex solution in 450 µl of prewarmed Opti-MEM supplemented with 1% (vol/vol) FBS. Let the solution sit at room temperature for an additional 10 min.

▲ **CRITICAL STEP** The addition of 1% (vol/vol) FBS here has been shown to enhance transfection efficiency in BMSCs (data not shown). If transfection is carried out on other primary cell lines, the effect of low amounts of serum should be empirically determined first.

(B) Two-part mixing with salt-free buffer

(i) To 47.5 µl of HEPES buffer (20 mM HEPES (pH 7.4)), add 2.5 µl of pDNA (0.4 mg ml^{-1}). Mix and allow the pDNA to equilibrate for 5 min at room temperature. (The final pDNA concentration per well in this setup is 2 µg ml^{-1}.)

(ii) To 47.5 µl of HEPES buffer, add 2.5 µl of bPEI25 (1 mg ml^{-1}); pulse-vortex to mix briefly. Let it sit for 5 min. The final polymer concentration per well is 5 µg ml^{-1}.

(iii) Form a complex by adding 50 µl of the diluted DNA solution in Step 17B(i) to 50 µl of the diluted polymer solution in Step 17B(ii); mix by vortexing for 5 s.

(iv) Incubate at room temperature for 25 min.

(v) Dilute the polyplex solution in 400 µl of prewarmed Opti-MEM. Let the solution sit at room temperature for an additional 5 min.

▲ **CRITICAL STEP** The final concentration of polymer listed above is 5 µg ml^{-1}, which may or may not be ideal for every cell line. If cell viability is markedly reduced at this concentration (<60%), lower the amount of polymer, but maintain the same polymer-to-DNA weight ratio.

▲ **CRITICAL STEP** The polymer-to-DNA weight ratio listed above is 2.5, which we have found to work well for both fibroblasts and bone marrow cells. However, the optimal weight ratio for other cell lines may be different and need to be empirically determined by transfection with complexes formed at various weight ratios (i.e., weight ratios of 1.25, 2.5, 5 and 10, or an N/P range from 10 to 80). The upper limit to this range is effectively determined by the toxicity of the polymer and the sensitivity of the cells to the polymer.

18| Aspirate the cell culture medium from each well and add the entire 500 µl of diluted polyplex transfection mixture directly to the cells.

▲ **CRITICAL STEP** Steps 17 and 18 should not exceed 30 min.

19| Gently agitate the plates and allow the complexes to equilibrate for 5 min in the incubator (37 °C).

? TROUBLESHOOTING

20| Force the complexes onto the cell surface at the bottom of the plate by centrifuging the plate at 210*g* for 5 min at room temperature in a microplate adaptor rotor. Set acceleration and braking to 1.

▲ **CRITICAL STEP** Ensure that plates are properly balanced before loading them into the centrifuge. Some cells may be sensitive to sudden and excessive force; thus, gentle acceleration and deceleration is recommended to minimize *g*-force shock.

21| Gently remove the plates from the centrifuge, being careful not to disturb the medium; return the plates to a 37 °C incubator.

? TROUBLESHOOTING

22| After 4–6 h, remove the transfection mixture by aspiration and replace it with cell culture medium. If desired, wash cells twice with cell culture medium to sufficiently remove complexes.
▲ **CRITICAL STEP** The incubation time can vary between 2 and 8 h. Centrifugation forces complexes to the bottom of the plate and onto the cell surface; thus, as little as 1 h can be allotted for transfection. If cells begin to show toxicity after 2 h, remove the complexes and replace them with culture medium. It is not recommended to leave complexes in for more than 16 h, as complexes will destabilize and aggregate, resulting in toxicity with lowered transfection efficiency.

23| After 24–48 h, quantify *GFP* expression for transfection efficiency by FACS analysis.
? TROUBLESHOOTING

FACS analysis ● TIMING 20 min (plus instrument time)
24| Aspirate the culture medium from cells. Wash cells three times with 500 µl of CMF-HBSS.
? TROUBLESHOOTING

25| Detach the cells by adding 100 µl of clear trypsin-EDTA (0.05% (wt/vol), without phenol red) to each well.

26| Allow the trypsin to equilibrate across the well surface for 2 min, and then agitate loosely attached cells by tapping on all four sides of the plate. Visualize the cells under a microscope to monitor the extent of detachment.

27| Once cells have detached from the surface, stop the trypsin activity and fix the cells by adding 100–150 µl of 3.7% (vol/vol) formalin in clear HBSS.

28| Analyze the cells on a flow cytometer. Excite GFP with an argon laser (488 nm) and detect it in FL1 channel. Adjust the voltage such that the distribution peak is between 10^0 and 10^1.
▲ **CRITICAL STEP** Be sure to set up a negative control by transfecting cells with a pDNA that does not contain *GFP* (null). Toxicity can induce autofluorescence and result in the overestimation of transfection efficiency.
■ **PAUSE POINT** If samples are not to be assayed immediately, store the fixed cells in a 4 °C refrigerator. Fixed cells may be stored for up to 1 week. Prolonged storage in the presence of formaldehyde is not recommended.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
4, 14	Cells are not attaching to surface	Plate surface not conducive to attachment	Ensure that plates are tissue culture treated. Alternatively, try plates treated with poly-L-lysine
	Low percentage of cell attachment	Prolonged handling of cells outside the incubator	Reduce the batch sizes such that they can be done in ~30 min from start to finish
		Cells are thawed for too long	Equilibrate the surface of the culture substrate with cell culture medium for 2–4 h
8	Cells are difficult to detach	Insufficient washing; wash buffer contains divalent cations	Ensure that the wash buffer is free of divalent cations. Perform additional washes and extend the wash time
		Strongly adhering cells	Increase the strength of trypsin from 0.05 to 0.25% (wt/vol); incubate at 37 °C for 2–5 min; flush the cells by gently pipetting up and down
14	Cells aggregate in the center of the well	Failure to distribute cells evenly across the surface	Monitor the attachment closely under the microscope. Agitate the plate frequently (every 5 min) to prevent cells from collecting at the center of the well

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Possible solution
	Cells aggregate in patches	Cells are damaged during treatment with trypsin	Shorten the duration of trypsin treatment; dilute trypsin with HBSS; tap on all sides of the flask gently; if you are unable to detach cells completely, it is best to minimize exposure to trypsin and seed healthy cells, albeit at lower density, than to have a higher density with patches of aggregated cells
16	Cells are slow growing	Seeding density is too low	Cell-to-cell contact is required for efficient growth. If initial seeding density falls below 20%, growth rate may be compromised. Try lowering the split ratio to increase cell concentration
19, 23	Toxicity after exposure to complexes	Complexes aggregate Concentration of polymer too high; complexes have destabilized	Monitor the complex preparation time during each step closely. The time between Steps 16 and 19 must not exceed 30 min Perform optimization by testing various concentrations of polymer (as a function of the N/P ratio) to determine an acceptable range Reduce incubation time with complexes Toxicity is a function of cell density and polymer concentration; try transfecting at a higher cell density Supplement transfection medium with 1% (vol/vol) FBS
21	Toxicity	Centrifugation speed is too high; centrifuge is not balanced	Cells may be sensitive to excessive force Balance the centrifuge Reduce centrifugation force down to the 120–180*g* range
		pDNA is immunogenic	Typical mammalian expression plasmids contain unmethylated CpG dinucleotides, which are known to induce the innate immune response via TLR9 receptors. Try using a minicircle pDNA devoid of the bacterially derived sequences
24	Low transfection	Cells are high passage	For fibroblasts, passages higher than 40 are less metabolically active and would start becoming senescent. Obtain a new batch of cells with a lower passage number
		Cells are slow dividing	Previous passage may be overgrown. Subculture cells for another passage to re-establish log-phase growth
	Loss of transgene expression over time	pDNAs lack replication and partitioning elements for maintenance in mammalian cells	Try different vector constructs such as the pEPI-1, which contains an S/MAR sequence Replace the expression vector with a minicircle DNA devoid of bacterial-derived sequences

● TIMING

Steps 1–5, revive frozen cells cell stock for subculturing: 5–7 d
Steps 6–16, cell seeding for transfection: 30–40 min for seeding (plus 1–2 d until cells are ready for transfection)
Steps 17–23, preparation of bPEI25/pDNA polyplexes for transfection: 30 min (plus 1–2 d to allow reporter gene expression)
Steps 24–28, FACS analysis of *GFP* expression: 20 min to detach cells into suspension (plus 1–4 h to run samples through the cytometer, depending on the number of samples)

ANTICIPATED RESULTS

Two of the most crucial parameters in complex formation that can be easily manipulated are volume of solution and incubation time for complexes. A smaller volume favors more frequent intermolecular interactions, effectively reducing the

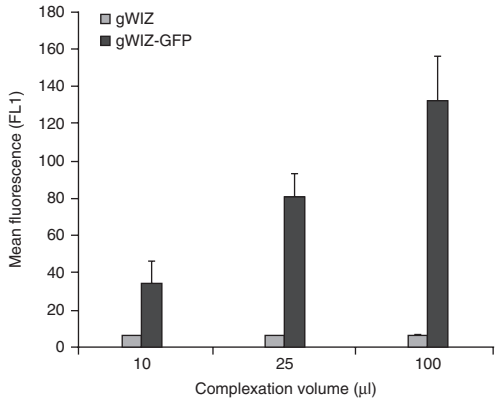

Figure 2 | Effect of complexation volume on transfection efficiency. Results were obtained from the transfection of NHFF in 12-well tissue culture plates where final transfection volume is 1,000 µl (complexes + medium). gWIZ and gWIZ-GFP refer to complexes with a control (non-*GFP* expressing) plasmid and an *GFP*-expressing pDNA, respectively. A positive correlation can be seen between transgene expression (mean GFP fluorescence) and volume of complexes. Results are means ± s.d. and are representative of two different experiments done in triplicate.

maturation time for complex formation—this may also accelerate the formation of aggregates, reducing overall transfection. A positive correlation can be seen between complexation volume and transfection efficiency in **Figure 2**. If toxicity is observed after complexes have been added to the cell, this could be the result of either destabilization of complexes/aggregates or the addition of an excessively high dose of polymer. Resolve the first issue by increasing the complexation volume or shortening the incubation time before attempting to lower the polymer concentration or the polymer-to-DNA weight ratio, as the former efforts will be more effective in optimizing transfection efficiency. As time is a crucial factor in the maturation and stability of complexes, assembled complexes left sitting for more than 30 min will gradually lose transfection efficiency.

The optimal polymer-to-DNA ratio for transfection will be an equilibrium between protection, condensation, dissociation and toxicity (for practical purposes, we refer to the ratio in terms of weight of polymer to DNA). As described earlier, the optimal ratio of polymer to DNA for transfection will be in excess of the ratio at which full condensation occurs. The presence of unbound free polymer is essential for overcoming the inhibitory effect of cell-surface GAG. However, too much free polymer may lead to additional toxicity, reduced uptake and lowered transfection efficiency. **Figure 3** shows transfection of fibroblasts at different polymer-to-DNA weight ratios. The weight ratio of 2.5 (effectively a N/P ratio of ~19) is optimal for transfection in fibroblasts. At a weight ratio of 5, the level of transgene expression is significantly reduced.

Once complexes are added to the cells grown in aqueous medium, a set incubation time is required to allow the complexes to settle down onto the bottom of the plate by gravity. **Figure 4** shows the level of transgene expression as a function of incubation time. Without centrifugation (0*g*), transfection increases with incubation time and peaks at 6 h, but the mean GFP fluorescence never reached the level achieved by centrifugation (210*g*). Further, transfection efficiencies after centrifugation were comparable between 1 and 6 h of incubation, suggesting the majority of the complexes have been effectively spun onto the cell surface. In short, transfection can be performed in as little as 1 h. Longer incubation (24 h) significantly reduced transfection efficiency presumably as a result of toxicity and loss of utility from the destabilized aggregates of complexes. Thus, centrifugation is a simple and easily accessible step that works analogously to magnetofection in forcing the complexes onto cell surface quickly, bypassing the diffusion barrier to minimize the incubation time.

Complex preparation using the direct mixing method in buffered saline followed by incubation in Opti-MEM (with brief centrifugation) seemed to be the best method for transfection of NHFFs and BMSCs. Typical transfection efficiency in NHFFs using the method outlined here is around 30–35%, but it can range from 13 to 60%. The lower end

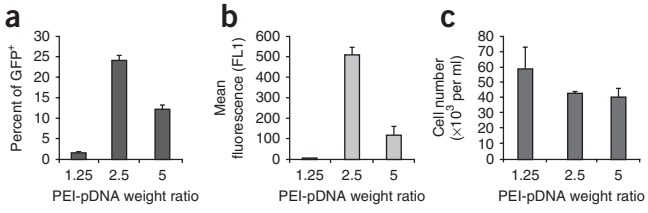

Figure 3 | Effect of PEI-to-pDNA weight ratios used in complex formation on transfection efficiency. Results show transfection of NHFF with bPEI25 complexes in a 24-well plate format. (**a**–**c**) Transfection results are assayed by flow cytometry for *GFP* expression and are summarized as the GFP$^+$ cell population (**a**), mean GFP fluorescence per cell (**b**) and cell viability (**c**). At the final pDNA concentration of 2 µg ml^{-1}, the polymer-to-DNA weight ratio of 2.5 (approximate N/P ratio of 19.4) was most efficient. Results are means ± s.d. from five different experiments done in triplicate.

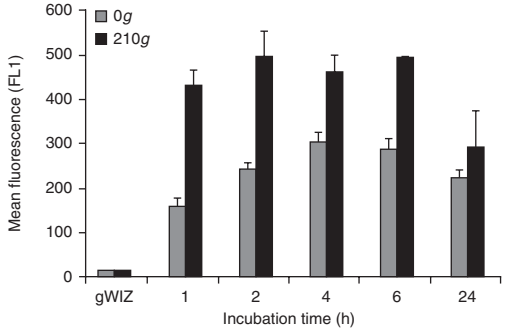

Figure 4 | Effect of centrifugation and incubation time on transfection efficiency. Results (mean GFP fluorescence per cell from flow cytometry) are shown for transfection of NHFF with bPEI25 complexes in a 24-well format. The control gWIZ polyplex is incubated for 6 h, whereas the gWIZ-GFP complexes were incubated for the indicated time periods. The cells were either incubated without centrifugation (0*g*) or with centrifugation (210*g*). The mean GFP fluorescence of cells increased with incubation time in the absence of centrifugation. With centrifugation, the mean GFP fluorescence was significantly higher across all incubation time frames with comparable levels from 1 to 6 h, suggesting that the majority of the complexes are effectively spun down onto the cell surface. Incubation with the complexes for 24 h markedly reduced transfection efficiency, presumably owing to toxicity from destabilized complex aggregates. Results are means ± s.d. and are representative of four different experiments done in triplicate.

of the spectrum is typically the result of slow-growing cells, because of low starting density, aging culture or high passage. Typical transfection efficiency for BMSCs ranges from 8 to 12%, and transfection efficiency up to 20% can be achieved with optimal culturing conditions.

In summary, the utility of bPEI25 as a transfection agent can be enhanced by updating transfection methods with the optimization procedures outlined here, thus providing an inexpensive alternative to commercial reagents commonly used for genetically manipulating the physiology of cultured cells.

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