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Investigating and Engineering the Delivery of DNA Drugs to the Nucleus

by

Richard Nathan Cohen

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Bioengineering

in the

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of the

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AND

ACKNOWLEDGEMENTS

I will never be able to show the appropriate amount of gratitude for all the people that have had an impact on my time in graduate school in a few short paragraphs but I will do my best and am very sorry if I accidentally omit anybody. I feel very fortunate that I have met such smart, interesting, passionate and fun people and know that my experiences here would never have been the same without them. We had some great times and some challenging times but I learned a lot from both and have developed much more as person than I ever would have expected when I packed my bags and moved to California.

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Taylor and Jeffrey Lowers in the UCSF Bioengineering office who tirelessly put up with my constantly late paperwork.

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Investigating and Engineering the Delivery of DNA Drugs to the Nucleus

by Richard Nathan Cohen

ABSTRACT

Non-viral gene therapy vectors have not yet achieved the gene transfer efficiency of viral vectors but continue to be an attractive alternative to viruses due to reduced toxicity and ease of preparation. Successful improvements to non-viral vectors will likely require rationally designed strategies that consider the many barriers that nucleic acid drugs must overcome, including distribution to target organs, internalization into cells, endosomal escape, transport in the cytoplasm, and nuclear uptake. Studies that increase our understanding of these barriers will therefore also represent a significant contribution to the development of new vectors.

This dissertation presents a series of studies related to the intracellular delivery of plasmid DNA to the nucleus. First, a new strategy was introduced to increase the cytoplasmic mobility of plasmid DNA: Biomolecular Adaptor for Retrograde Transport (BART) peptides designed to hijack endogenous molecular motors. Proof-of-concept experiments showed that a BART peptide can bind to a dynein light chain and that BART-bound cargo can be actively transported along microtubules by dynein in a reconstituted system. Second, an assay was developed to quantify the amount of plasmid DNA that is delivered to cell nuclei using various delivery conditions. Application of this assay revealed that cells transfected with Lipofectamine™ lipoplexes or polyethylenimine polyplexes contained between 75 and 50,000 plasmids/nucleus and that

lipoplex-delivered plasmids were more efficiently expressed, based on protein expression per plasmids in the nucleus, than polyplex-delivered plasmids. Third, optimized conditions were established for a robust high throughput assay to screen for small molecule enhancers of transfection. Enhancer molecules discovered with this screen could help to elucidate rate-limiting steps in the transfection process and could also be used as adjuvants in gene therapy formulations. These studies summarize my efforts to design new tools and gauges to improve both the design of and our understanding of gene vectors.

In addition, the final report of a field study in the Dominican Republic to assess the feasibility of needle-free injection technology for that country's immunization program is included at the end of this dissertation. This report presents recommendations on how current devices could be improved and how the technology could be implemented.

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CHAPTER 1: Introduction

This chapter contains material reprinted from the review article "Molecular motors as drug delivery vehicles" by Richard N. Cohen, Mark J. Rashkin, Xin Wen, and Francis C. Szoka in Drug Discovery Today: Technologies, 2(1) (2005) 111-118 with permission from Elsevier.

1.1 OVERVIEW

Gene therapy can generally be defined as the correction of a disease phenotype through the introduction of new genetic information into an affected organism. With advancements in molecular pathology, genomics, and expression profiling, many targets for gene therapy have emerged and the ability to modulate these genes was once thought to be a straightforward and simple approach to treating disease. In most cases, however, this treatment requires the delivery of nucleic acid (DNA or RNA) into the organism's cells and/or the nuclei of these cells, which have evolved specific mechanisms to prevent that very transfer of genetic information from occurring. Viruses have evolved mechanisms to overcome many of these barriers to delivery and have thus proven to be excellent carriers of therapeutic nucleic acids into mammalian cells. However, limitations of viral vectors, including toxicity, immunogenicity, difficulty in generating high titers, and genetic instability have led to an increasing amount of research and clinical trials using non-viral delivery with synthetic vectors. Nevertheless, non-viral vectors, including polymers and liposomes, have not been able to achieve the gene transfer efficiency of their viral counterparts.

The focus of this dissertation is on strategies to improve and measure the delivery of plasmid DNA to the nucleus of transfected cells with non-viral vectors. The most promising approaches to improve the efficiency of non-viral gene therapy will most likely be devised by logical consideration of the many barriers that nucleic acid must overcome to reach its target site. Chapter 1 briefly introduces the topics of intracellular and intranuclear DNA delivery and describes the potential utility of molecular motors as drug delivery vehicles. Chapter 2 introduces a new approach to improve the intracellular mobility of plasmid DNA or other exogenous cargo by hijacking molecular motors inside of cells. Chapter 3 details a quantitative assay to measure the amount of plasmid DNA that is delivered to cell nuclei and the application of this assay to compare the nuclear delivery efficiency of two commonly used transfection agents. Chapter 4 describes the development of a high throughput screen for small molecular enhancers of gene transfer that could also help to elucidate rate-limiting steps of the gene transfer process. Finally, Chapter 5 provides a context for the results presented in the previous chapters and discusses the future of non-viral gene therapy. The addendum contains a report, written from data collected during a field research trip to the Dominican Republic concerning the feasibility and patient acceptance of needle-free delivery of injectable medications or vaccines. The transmission of diseases between patients or between patients and health care providers by the re-use of needles and syringes or via accidental sticks from a used needle, could be greatly reduced by the introduction of needle-free single use devices. My field research indicates that such devices would be accepted by healthcare providers and by patients if current prototypes were redesigned to make them cheaper and easier to use.

1.2 DELIVERY OF DNA TO THE NUCLEUS- INTRACELLULAR BARRIERS

A majority of the drugs on the market today target cell surface receptors which are easily accessible on the outside of cells. A drug can act upon these receptors if it can reach the target cell before being metabolized or cleared from the body. For a drug to reach intracellular targets, such as mitochondria, the endoplasmic reticulum, and the nucleus, it must traverse multiple additional barriers. For example, for DNA to be used as a drug in gene therapy, it must overcome multiple cellular barriers to reach its target: the nucleus. These barriers, diagramed in Figure 1-1, have been well reviewed elsewhere [1-4] and are briefly discussed below.
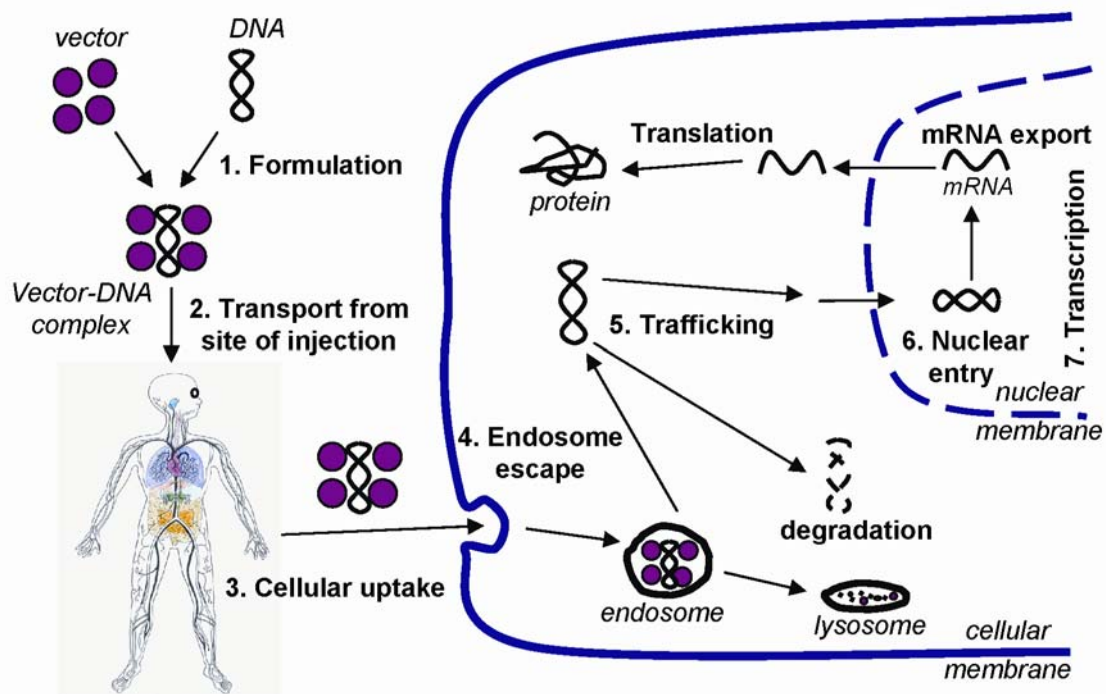
Figure 1-1 Barriers to gene delivery.
The obstacles that must be considered in the rational design of non-viral gene delivery vectors are indicated in bold.

Following formulation (step 1) and transport from the site of injection (step 2), requirements for all drug carriers, DNA must be taken up into the cells (step 3). For most DNA vectors, this process occurs by endocytosis [5]. Following endocytosis, the DNA will reside in an endosome and must escape (step 4) before the vesicle is trafficked to the lysosome, which occurs within 60 minutes [6]. In lysosomes, the cell will attempt to degrade the DNA with nucleases and a decreased pH. Many DNA carriers mediate escape from the endosome by responding to change in pH (polyamines and pH-sensitive lipids or polymers) [6, 7] or by interacting directly with the anionic lipids that make up the endosomal membrane (cationic lipids) [8].

Following endosomal release, the DNA will reside in the cytoplasm and must traffic (step 5) to the nucleus before degradation or inactivation by cytoplasmic nucleases. Despite evidence that the mobility of DNA in the cytosol may be a barrier to gene transfer, there has been little effort to overcome the barrier of transporting DNA towards the nucleus [9-11]. Previous studies have suggested that the half life of DNA in the cytoplasm is less than 90 minutes [2]. The diffusion of plasmid DNA has been shown to be effectively zero in the cytoplasm [12] due to the large side of the plasmid itself (~10,000 times larger than a small molecule drug). In reporter gene expression studies, 95-100% of cells whose nuclei were microinjected with DNA were efficiently transfected compared to 0.001% of cells with DNA microinjected into the cytoplasm [13]. Transfection efficiency dropped from 56% when the DNA microinjection was made near the boundary of the nucleus to 8% when 60-90 µm distal to the nucleus [14]. Efforts to

use molecular motors to improve the mobility of plasmid DNA in the cytoplasm are discussed in section 1.4 and in Chapter 2.

As mentioned above, for a gene to be expressed from delivered plasmid DNA, the DNA must enter the nucleus (step 6). This is another nontrivial problem since the portal into the nucleus, the nuclear pore complex, restricts access of molecules larger than 39 nm [15]. One scenario for how DNA can enter the nucleus is by utilizing the active nuclear import system, which has been suggested as a possible mechanism if the plasmid contains sequences that bind to certain transcription factors (TFs) [16, 17] or if the plasmid is conjugated to a nuclear localization signal (NLS) peptide [18-20]. In the absence of TF-binding sequences or NLS peptides, plasmid DNA may enter the nucleus by passive entrapment within a newly formed nucleus following the breakdown and reformation of the nuclear membrane that occurs during cell division, which has been suggested to be the primary means of nuclear entry in rapidly dividing cells [21], or by a yet undiscovered mechanism [22]. An assay for quantifying how much plasmid DNA reaches the nucleus of transfected cells is presented in Chapter 3.

Other barriers to gene expression from delivered plasmids include transcription (step 7) of the gene sequences and vector unpacking. Transcription requires an appropriate mammalian gene expression construct, with associated promoter and enhancer sequences. Recent studies have also emphasized the effect of subnuclear localization of plasmid DNA on gene expression and how this localization may be controlled [23, 24]. Vector unpacking, which has been shown to be an important step in

transfection [25, 26], may occur in either the endosome, the cytoplasm, or the nucleus. The timing of vector unpacking is an important consideration because plasmid release from the vector too early could leave the plasmid vulnerable to nuclease degradation and plasmid release too late or not at all could interfere with the transcription of the delivered gene.

1.3 VECTORS FOR NON-VIRAL GENE THERAPY

The many currently available non-viral gene therapy vectors, including lipids [4, 27, 28], polymers [29-31], and peptides [32-34] have been extensively reviewed elsewhere. An ideal non-viral delivery system would address all of the barriers described above, as viruses do, though it is also important to consider that an overly complicated synthetic system may hinder its commercial viability. In the design of non-viral delivery vectors, one should therefore consider which steps in the transfection process are rate-limiting and to focus efforts on addressing these steps. Unfortunately, it is not well understood which steps are rate-limiting and the rate-limiting steps are also not necessarily the same in all cell types and with all gene therapy vectors. Therefore, the development of new vectors would also benefit greatly from studies designed to investigate the relevance of each of these steps.

1.4 MOLECULAR MOTORS AS DRUG DELIVERY VEHICLES
1.4.1 Overview

Over the past few years there has been a significant increase in the understanding regarding the function and role of molecular motors in the cell [35]. It is now evident that these motors are required for many cellular processes, including vesicle transport,

mitotic spindle formation, and cell motility [36]. Recently, researchers have begun to shift their efforts towards developing applications that utilize this technology in novel ways, such as part of nanomachines [37, 38] and for the delivery of genes or drugs to the nucleus of cells or to the central nervous system. A successful implementation of these latter applications is on the path to the design of an efficient viral mimic since many viruses achieve their high level of infection by hijacking cellular motors during infection [39]. In this section we describe protein motors and their potential applications as a drug delivery vehicle. In addition, we will introduce a new technology that enables the attachment of cargo to motors for gene and drug delivery, the biomolecular adapter for retrograde transport (BART).

1.4.2 Molecular Motors

The cytoplasm of a eukaryotic cell is a crowded, viscous milieu consisting of proteins, organelles, and RNA, which limit the diffusion of macromolecules. Although the viscosity of the cytoplasm is similar to that of water, macromolecules greater than 5,000 kD and subcellular structures such as vesicles, diffuse 500-1000 times slower in the cytoplasm compared to an aqueous solution [40]. This is a result of molecular crowding and interactions with organelles and immobile cytoskeletal proteins. In order to generate and maintain intracellular organization, cells have devised strategies of moving larger cellular components (cargo), such as vesicles and mRNA, from place to place as needed [41, 42]. There are three types of cytoskeletal motors that are key to transport inside cells: kinesins, dyneins, and myosins. For short distance transport, myosin carries its cargo along short actin filaments, which are found throughout the cell but are most highly

concentrated near the cell membrane [43]. For long distance transport, kinesins and cytoplasmic dynein carry cargo along microtubules, which are long polarized filaments with their plus (+)-ends located in the cell periphery and their minus (-)-ends located at the microtubule organizing center (MTOC) near the nucleus [44]. Long distance transport by microtubular motor proteins is especially important in the long axons of nerve cells in which microtubles are oriented with the minus-end pointed towards the cell body and the plus-end oriented towards the axon terminal. Microtubular motor proteins are unidirectional in that they move exclusively in one direction along microtubules: most kinesins carry cargo towards the microtubular plus-end in the cell periphery or axon terminal (anterograde); all dynein and a few kinesins carry their cargo to the minus-end of microtubules near the nucleus or cell body (retrograde).

All three of these motors are protein complexes which contain multiple domains responsible for cargo binding and motor function. The 'head' of the complex contains the motor domain that provides the motion along the tracks while the "tail" of the complex contains the subunits responsible for cargo binding and regulation. The 'head' group is thus considered the engine of the motor and is fueled by ATP hydrolysis. The hydrolysis of ATP also provides the motor complex a mechanism for movement, during ATP hydrolysis the protein undergoes small changes in structure that are amplified throughout the complex. Simplistically, the motor walks along the cytoplasmic track in an iterative cycle of binding, hydrolysis, and unbinding steps by the motor 'head'. The motor functions (ATP hydrolysis cycle, force generation, step size, etc.) of myosin [43] and kinesin [45] have been studied in detail and the specifics of dynein motor function

are also beginning to be discovered [46]. The mechanism of motor-cargo binding and motor regulation in the "tail" region is beginning to be understood [41, 42] yet many questions still remain as to the logistics of cargo transport. It is also now clear that myosin, kinesin, and dynein interact with each other either directly or indirectly, but it is still unknown how the motors determine which cargo to transport and when to transport the cargo to its proper location within the cell [47].

In addition to the microtubular transport motors discussed thus far, cells contain many other molecular motors that perform a variety of functions. For example, F_1-ATPase is a rotary motor that synthesizes ATP from ADP and is capable of producing ~40 pN·nm of torque [48]. The bacteria flagellar motor provides motion that allows bacteria to swim through their environment [49]. Helicases are motors that unwind the double helix of DNA, which allows for replication and/or transcription [50]. DNA and RNA polymerase are motors that move along DNA and RNA while they synthesize growing nucleic acid strands [51].

1.4.3 Bioengineered Molecular Motors

As more is learned about how molecular motors function, it is becoming feasible to harness the power of these machines to perform functions other than those for which they evolved to perform. The first report of modifying molecular motors to power nanomachines in synthetic environments used the F_1-ATPase motor protein. Fluorescently labeled actin filaments were attached to the F_1-ATPase motor, and in the presence of ATP the filaments rotated, creating a propeller-like machine that converts chemical energy into mechanical energy [52]. This design has since been modified to

incorporate nanofabricated inorganic components and a zinc-dependent on/off switch [53, 54]. Another example of an engineering applications of molecular motor involves the design of molecular shuttles based on kinesin that can either transport cargo in synthetic environments along microtubular tracks or carry microtubules around nanoscale "roundabouts" [55, 56]. Potential applications for these systems include miniaturized analytical systems, adaptive and self-healing materials, and directed molecular assembly [57]. Motors have also been engineered to modify their speed, directionality, and cargo binding. For example, the speed of myosin has been adjusted by incorporating artificial lever arms of various lengths into the complex [58], and Manstein and coworkers have reversed the directionality of myosin by rotating the direction of the motor's lever arm 180° [59].

1.4.4 Molecular Motors for Gene and Drug Delivery

It is possible that molecular motors could also be designed to perform therapeutic functions inside of cells, such as improving non-viral gene therapy and interneuron delivery. We believe that molecular motors can be used as an active transport mechanism to move DNA, effectively bypassing the resistance to cytoplasm movement (See Appendix 1-1 for a discussion on the forces required to transport DNA in the cytoplasm). There are at least three possible means of using molecular motors to actively transport DNA or other drugs through cells: (i) Lay new tracks in the cell with one end near the nucleus and add a synthetic motor designed to carry DNA along these tracks toward the perinuclear ends. (ii) Use tracks already available in the cell (ie. microtubules) but add a synthetic motor that can carry DNA along these tracks. (iii) Use a motor already in the

cell that moves along tracks already in the cell and connect DNA to this motor via a molecular adaptor. We designate a synthetic adaptor that is capable of linking cargo to a motor for the purpose of transporting that cargo to the interior of the cell a Biomolecular Adaptor for Retrograde Transport or BART. Our lab is attempting to use molecular motors as a DNA delivery vehicle using the latter two approaches discussed above: a synthetic motor and BART (Fig. 1-2).
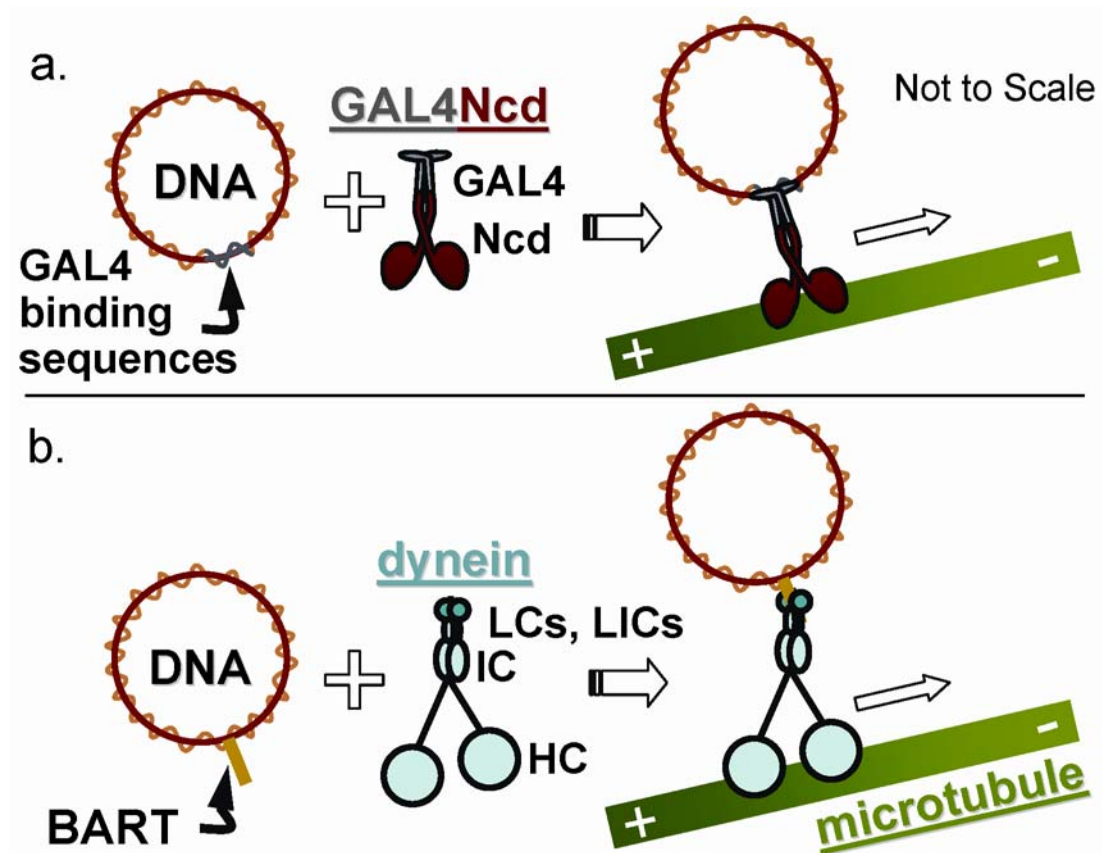
Figure 1-2 Strategies to use molecular motors to actively transport DNA to the nucleus.
a) Engineered molecular motor carries DNA along microtubules. The chimeric protein consists of a microtubular minus-end directed motor protein, such as non-claret disjunctional (ncd), and a DNA-binding domain, such as that from GAL4. The plasmid contains repeats of the DNA-binding proteins recognition sequence. b) A biomolecular adaptor for retrograde transport (BART) links DNA to endogenous dynein which then carries DNA along microtubules. BART is covalently linked to plasmid DNA. Dynein subunits: LCs, light chains (LC8, tctex-1, roadblock); LICs, light intermediate chains, IC, intermediate chain; HC, heavy chain.

The first approach that has been attempted in our lab involves creating a fusion protein that contains a GAL4 DNA binding domain and a minus-end directed motor

domain. We used the non-claret disjunctional (Ncd), the first discovered and most extensively studied minus-end directed kinesin motor protein [60], as the motor to assess the potential application of DNA translocation via motor proteins. Our hypothesis is that a molecular motor engineered to have DNA-binding capabilities will improve transfection by transporting DNA to the vicinity of the nucleus. As proof that the cargo-binding unit of Ncd can be replaced with a novel cargo-binding unit, Vale and coworkers engineered Ncd to carry lipid vesicles by fusing a lipid-binding domain to Ncd's motor-stalk domains [61]. Our system has two-components, GAL4-Ncd and a luciferase reporter plasmid, containing GAL4 recognition sequences (Fig. 1-2a). Standard microtubule gliding assays demonstrated that the GAL4-Ncd chimera has comparable motor activity to the N-terminal truncated Ncd protein. When the reporter plasmid (pGAL4) was transfected into CV-1 cells in the presence of the fusion motor protein the luciferase activities were increased nearly 6-fold (Fig. 1-3a). This transfection enhancement was not observed for control plasmids lacking the appropriate binding sequences (pControl).

To explore whether intact microtubules were required for the observed increase in transfection efficiency, we disrupted the cellular microtubule network through the use of nocodazole or taxol and observed the increase in transfection in fusion protein treated cells was reduced (Fig. 1-3b). This suggests that the increase in transfection was associated with the ability of the motor to translocate DNA along the microtubule pathway. To prove that the active transport of DNA is the cause of the increased

transfection, future experiments should examine whether the fusion protein binds specifically to the reporter plasmid and if the motor function of Ncd is necessary.
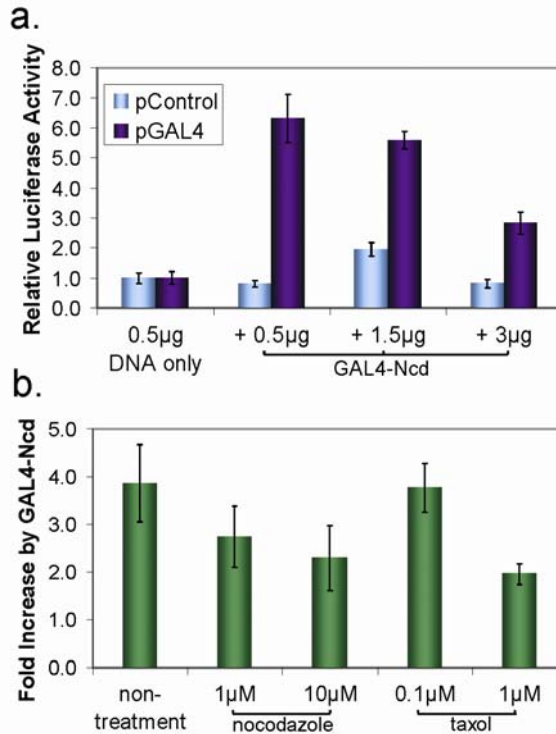
Figure 1-3 GAL4-Ncd enhances the transfection of GAL4 reporter plasmid in a partially microtubule-dependent manner.
a) Transfection complexes prepared by incubating 0.5 µg plasmid with the indicated amounts of GAL4-Ncd in binding buffer for 15 minutes and then with DOTAP liposomes for an additional 15 minutes.   CV-1 cells were incubated with transfection complexes for 4 hours and grown in growth medium for an additional 20 hours. pGAL4, luciferase plasmid containing 5 repeats of GAL4 recognition sequence in non-coding region; pControl, luciferase plasmid lacking GAL4 recognition sequences. b) DOTAP transfection same as in A. Cells were treated 1 hour prior to transfection with indicated amount of microtubule-disrupting drugs.

An alternative gene delivery method currently under study in our laboratory is the use of BART, which is the subject of Chapter 2 of this thesis. BART is a synthetic adapter that links DNA or some other novel cargo to dynein (Fig. 1-2b). In designing a system that manipulates the intracellular environment, minimal perturbation of the cell is desired; therefore, this system, which uses an endogenous motor might have an advantage over the previously described GAL4-Ncd system. In this case, dynein is akin to a train

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traveling along rail tracks and the novel cargo is a passenger trying to get towards the nucleus. This is a similar approach to that employed by many viruses. For a full description of the BART system, refer to Chapter 2.

Molecular motors may also be applied as a drug delivery vehicle to the cell bodies of motor neurons by retrograde axonal transport following peripheral nerve injection [62]. Similar strategies have been studied with retrograde transport of gene therapy vectors made with herpes simplex virus-1 and rabies G protein-pseudotyped lentivirus [63, 64]. When these vectors were injected into either the footpad or sciatic nerve of mice and rats, reporter gene expression was observed in motor neurons and the root dorsal ganglion (RDG) of the spinal cord. Ultimately, a delivery vector that included a mechanism for transynaptic transport in addition to retrograde transport could deliver drugs directly to the brain following injection into remote and easily accessible parts of the body.

1.4.5 Conclusions

Molecular motors are highly efficient nanoscale machines evolved with the kind of ingenuity most engineers dream of being able to design. As our understanding of the structure and function of molecular motors expands, it becomes feasible to utilize the power of these machines for novel engineering applications. A number of outstanding issues remain. First, much more needs to be learned about how motors bind to specific cargo, regulate this binding, and control motor function to ensure appropriate transport of each cargo to an appropriate location at the appropriate time. As more is learned about the mechanisms of motor binding and regulation, the appropriate motor components

could be modified to carry novel cargos, as in the case of GAL4-Ncd, or the cargos could be modified to bind to a specific motor, as in the case of the BART system. Second, it remains to be seen if modified motors can function in a robust fashion in nanoscale devices or inside cells or if such applications will remain a laboratory curio. Engineered motors used in synthetic devices will likely have to be oriented in an organized pattern and be interfaced with inorganic components. Engineered motors used inside cells or molecules that target intracellular motors function in an extremely crowded setting where endogenous cellular components will potentially compete for the same tracks, motors, and/or regulatory proteins. Furthermore, for intracellular applications, it remains to be seen whether a molecular motor can be used to target large macromolecules to specific subcellular locations. Efficient intracellular targeting will require a greater understanding of how traffic is coordinated among the many "trains" and "train tracks" inside cells. In the non-viral gene therapy example discussed in this chapter, it must be shown if an engineered motor or a motor adaptor, such as BART, can actively carry large DNA plasmids to the nucleus and if so, how this will affect gene transfer efficiency.

The potential applications of molecular motors extends beyond those discussed here and may only be limited by the extent of our collective imagination. Anterograde transport by conventional kinesin could be used to transport novel cargo to the cell membrane for secretion. An appropriately shaped and oriented rod attached to a rotary motor could be used to propel a nanomachine through tissue. If this motor responded to a chemical gradient, the nanomachines could be targeted to a specific region of tissue such as a tumor or a site of damage. The forceful movement of a myosin's lever arm could be

used as a jack hammer to punch holes in membranes. A novel molecular motor could be used to traverse membranes by forming transient attachments to phospholipids head groups, much like a monkey swinging on vines in a forest. A DNA polymerase engineered to relay an electrical signal based on the base that it adds to a growing nucleic acid chain could be used for rapid DNA sequencing. The successful understanding and engineering of these motors may lead to applications and solutions to problems beyond those even imagined today. The horizons are broad and the ability to engineer these systems may soon create fact from science fiction.

1.5 APPENDIX 1-1
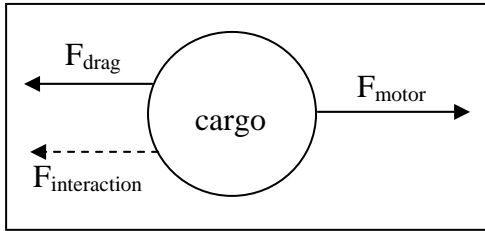
To transport every cargo in the cytoplasm, a motor protein must produce enough force to overcome the drag force on the cargo which includes molecular crowding effects and the binding to cellular components. The drag forces can be characterized by a drag coefficient (γ) which is related to the diffusion coefficient (D) according to the Einstein relation:

$$\gamma = \mathrm{kT}/D \qquad (1)$$

where kT is thermal energy (4.28×10^{-21} J at 37ºC). The drag force of a particle is related to the drag coefficient and the velocity ($\mathbf{v}$) of the particle by:

$$\mathbf{F_{drag}} = \gamma\mathbf{v}. \qquad (2)$$

In single-particle tracking studies in living CV-1 fibroblasts, 80 nm radius inert green fluorescent microspheres were observed to have a D of approximately 4×10^{-11} cm^2/s [40]. Using fluorescence recovery after photobleaching (FRAP), 6000 bp plasmid DNA was shown to have a similar D in HeLa cells [12]. To diffuse 10 µm (the approximate distance from the cell membrane to the nucleus in a 20 µm cell) would take, on an average, more than 100 minutes, although only a portion of the DNA would diffuse in the direction of the nucleus. Because the half-life of free plasmid DNA in the cytoplasm is 50–90 min [2] a significant amount of DNA that is taken into cells is degraded before reaching the nucleus if diffusion is the only means of mobility.

A D of 4×10^{-11} cm^2/s corresponds to a γ of 1.1×10^{-6} N·s/m and an $\mathbf{F_{drag}}$ of 1.1 pN if the DNA or microsphere were to be transported at a rate of 1 µm/s, which is approximately the observed velocity of kinesin- and dynein-mediated transport [44]. For reference, 1 pN is approximately the amount of force a laser pointer exerts on a projection screen, extremely small on a macroscopic scale but significant on the scale of molecular motors. Because the maximum force outputs of dynein and kinesin are 1.1 and 7.5 pN [47], respectively, it may be necessary for multiple motors to simultaneously exert a force on a plasmid in order to transport it in the cytoplasm, depending on its size. This could be achieved by adding multiple motor binding sites to the plasmid.

CHAPTER 2: Biomolecular Adaptors for Retrograde Transport (BART)- Active Transport by Dynein to the Nucleus

2.1 ABSTRACT

As our understanding of molecular motor function increases, we can begin to engineer new systems that utilize these machines in novel ways. We hypothesize that dynein-binding peptides sequences could be used to link a conjugated molecule or particle to dynein to allow for transport of novel cargo (eg. plasmid DNA) through the cytoplasm towards the nucleus. We synthesized selected sequences from proteins that were previously shown to bind to one of two dynein light chains, LC8 and Tctex, and designate these peptides as Biomolecular Adaptors for Retrograde Transport (BART). Using a peptide pulldown assay, BART1 was shown to bind specifically to LC8 while another peptide (BART1C) with two amino acids mutated from BART1 did not bind to LC8. BART2, which was designed to bind to Tctex did not bind to either light chain tested. To test the ability of BART1 to mediate transport by dynein of cargo conjugated to this peptide, *in vitro* bead motility assays were performed. At a ratio of ~10:1 dynein:bead, BART1-nanospheres were observed moving along microtubules in a dynein-dependent manner while no movement was observed for BART1C-nanospheres. At higher dynein:bead ratios, the two nanospheres moved at a similar frequency, likely due to nonspecific binding. These results represent the first demonstration of dynein-mediated bead transport using a peptide adaptor. With further development, this system

could be developed to carry plasmid DNA to the nucleus to improve the efficiency of non-viral gene therapy.

2.2 INTRODUCTION

The journey of DNA drugs from the outside of a cell to the nucleus requires crossing both the cellular and nuclear membrane and traversing the crowded cytoplasm. Although many strategies have been devised by scientists developing non-viral gene carriers to allow for internalization into cells, endosomal escape, and nuclear uptake [4, 65-69], there has been very little reported on efforts to improve the transport of DNA drugs through the cytoplasm. However, diffusion of DNA above 2 kb is severely restricted in the cytoplasm [12] and the presence of various nucleases in the cytoplasm limit the half-life of unprotected DNA to less than 90 minutes [14]. Many viruses utilize their host cell's native motility system to allow for active transport towards the nucleus in the interior of the cell [70-72] and a synthetic strategy that mimics this viral approach has the potential to greatly improve the efficiency of non-viral gene transfer [73].

Eukaryotic cells contain long proteinaceous filaments called microtubules that are oriented with one end (the plus-end) in the cell periphery and the other end (the minus-end) at the microtubule organizing center in the vicinity of the nucleus. Protein complexes called molecular motors carry various cargoes in a specific direction along the microtubules: most kinesins travel towards the cell periphery while all dyneins and some kinesins travel towards the cell interior. Some reports in the literature indicate that DNA delivered with polymers [74, 75] or liposomes [76] are naturally carried along microtubules by dynein, although it is likely that this movement occurs while the DNA is

inside an endosome. Regardless of how long the DNA resides in an endosome or where it is released from the endosome, at some point the DNA will be in the cytoplasm and improvement of its transport through the cytoplasm towards the nucleus might have significant impact on its delivery to the nucleus or at least will reveal the importance of the cytoplasmic barrier to efficient gene transfer.
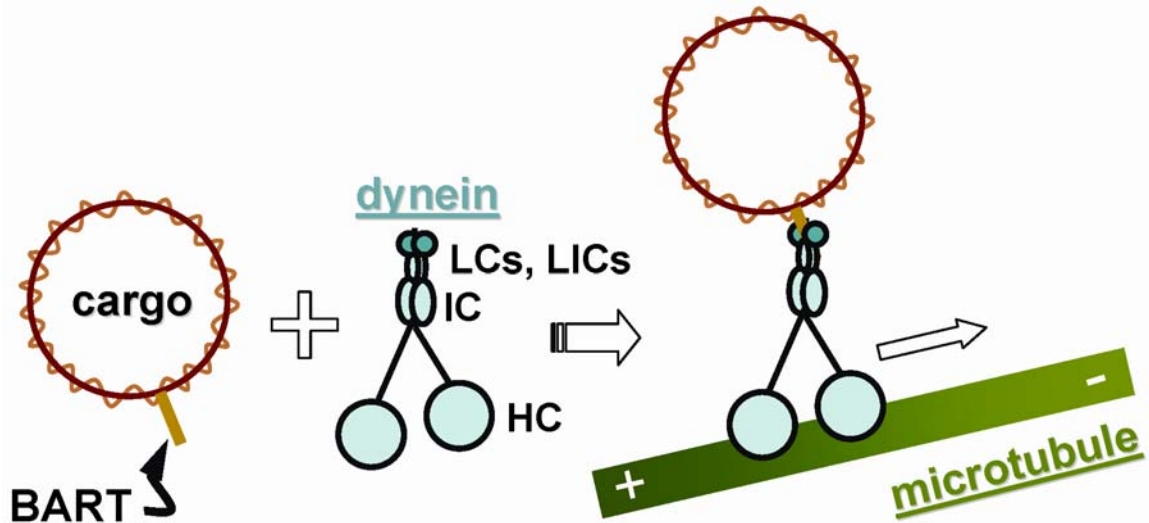
Figure 2-1 Diagram of proposed BART peptide system approach.
Biomolecular adaptor for retrograde transport (BART) peptides contain a sequence that binds to a cargo-binding subunit of dynein. When BART peptides are conjugated to a cargo particle or molecule, dynein will carry the cargo along microtubules towards the minus end which is located near the nucleus in most cells. Dynein subunits: LCs, light chains (LC8, tctex-1, roadblock); LICs, light intermediate chains, IC, intermediate chain; HC, heavy chain.

We have attempted a synthetic approach to target DNA to molecular motors that carry cargo along microtubules: peptides that binds to a light chain of dynein and can serve as a linker for novel cargo (Fig. 2-1). We refer to these peptides as biomolecular adaptors for retrograde transport (BART). We demonstrate the binding between a BART peptide and a dynein light chain and the transport of BART-bound beads along microtubules by dynein. Although some hurdles remain in the advancement of this technology, this approach has the potential to improve the transport of DNA in the cytoplasm and could improve the efficiency of non-viral gene therapy.

2.3 METHODS

2.3.1 Materials

Purified tubulin and flash-frozen bovine brain tissue were a generous gift from the laboratory of Stephen J. King at the University of Missouri-Kansas City. ATP was from Fisher Biotech (Pittsburgh, PA). AMP-PNP, hexokinase, PMSF, TAME, TLCK, TPCK, poly-L-lysine, and casein from bovine milk (technical greade) were from Sigma (St. Louis, MO). Pepstatin and leupeptin were from Roche (Indianapolis, IN). GTP was from Acros Organics (Morris Plains, NJ). Taxol (paclitaxel) was from MP Biomedicals (Solon, OH). hexokinase, protease inhibitors

2.3.2 Synthesis of Biotinylated BART Peptides

BART peptides, BART1, BART1C, and BART2 (Table 2-1), were synthesized with an Applied Biosystems (Foster City, CA) 341A peptide synthesizer using FMOC/DCC/HOBT chemistry and purified by HPLC. The peptides were biotinylated by reacting with EZ-link maleimide-PEO2-Biotin (Pierce, Rockford, IL) and unreacted biotin was removed by separation on a Superdex 75 column (Amersham, Pittsburgh, PA). The masses of biotinylated and unbiotinylated peptides were confirmed with a PerSeptive Voyager-DE MALDI-TOF mass spectrometer (Framingham, MA).

2.3.3 Purification of LC8 and Tctex1

The plasmid pET23b-LC8 encoding for a C-terminal His6-tagged rat LC8 was a gift from Dr. Ignacio Rodriguez-Crespo. LC8-His was expressed in E. coli BL21 DE3 and purified with a Ni^{2+}-NTA-agarose column (Qiagen, Valencia, CA). Fractions

containing LC8-His were further purified by size exclusion chromatography with a Superdex 75 column. pET23b-Tctex1 encoding for His6-tagged mouse Tctex1 was constructed by ligation of a PCR amplified fragment from a cDNA clone (ATCC Number 973480) with engineered NdeI and XhoI restriction sites and the pET23b vector backbone digested with NdeI and XhoI. Tctex1-His was expressed in E. coli BL21 DE3 and purified with a HisTrap FF column (Amersham, Pittsburgh, PA). Both LC8 and Tctex1 were stored as frozen aliquots in 20 mM ammonium bicarbonate, pH 7.8, 100 mM NaCl. Denatured and native protein size were determined by SDS-PAGE and analytical size exclusion chromatography, respectively. The secondary structure of each protein was assessed with a JASCO J715 circular dichroism instrument (JASCO, Easton, MD).

2.3.4 Streptavidin Pull-down Assay

Each peptide was loaded onto streptavidin-agarose resin (Invitrogen, Carlsbad, CA) in an empty Zeba™ spin column (Pierce, Rockford, IL). Peptide-loaded (and unloaded) streptavidin columns were incubated with LC8, Tctex1, or BSA for 1-1.5 hours at 4ºC. After several washes, retained proteins were eluted with a 4 minute incubation in 1M NaCl, 50 mM NaOH, pH 12.5 followed by neutralization in an equal volume of 100 mM Tris·HCl, pH 2.0. Elution fractions were detected by SDS-PAGE.

2.3.5 Dynein Purification

Dynein was purified from bovine brain tissue by a protocol modified from Schroer and Sheetz [77]. Protease inhibitors (100 µg/ml PMSF, 2 µg/ml leupeptin, 1

μg/ml pepstatin A, 10 µg/ml TAME, 10 µg/ml TLCK, and 10 µg/ml TPCK) were used in every step.

Forty grams of flash-frozen bovine brain tissue was thawed at 37ºC in homogenization buffer (PMEE [35 mM PIPES, pH 7.2, 5 mM MgSO$_4$, 1 mM EGTA, and 0.5 mM EDTA], 1 mM DTT, and 0.5 mM ATP) and homogenized on ice with a dounce homogenizer using one stroke of the A (loose) pestle and nine strokes of the B (tight) pestle. After a 5-minute incubation on ice to depolymerize microtubules, the homogenate was cleared by centrifuging at 33.5K x g (17.5K RPM in an SS-34 rotor, Sorvall Instruments, Dupont, Newport, CT) for 15 minutes at 4ºC and then ultracentrifuging at 200K x g (39K RPM in an SW41 Ti rotor, Beckman Coulter, Fullerton, CA) for 30 minutes at 4ºC. Native microtubules were spun down from the cleared homogenate by incubating with 1 mM GTP and 20 µM taxol for 30 minutes at 37ºC and ultracentrifuging at 200K x g (39K RPM in an SW41 Ti rotor, Beckman Coulter, Fullerton, CA) for 30 minutes at 31ºC.

Meanwhile, fresh microtubules were polymerized by incubating pre-purified tubulin (enough to have 0.25 mg tubulin/ml of cleared homogenate) with 1 mM GTP and 20 µM taxol for 15 minutes at 37ºC and then stored at room temperature. To bind dynein to the fresh microtubules, the cleared homogenate was mixed with the fresh microtubules along with a microtubule-binding cocktail (1 mM AMP-PNP, 10 mg/ml glucose, 4 mM MgSO$_4$, and 1 U/ml hexokinase) and incubated at ~30ºC for 20 minutes. The binding mixture was then layered on top of a 12.5%/25% sucrose cushion (containing 1 mM GTP,

20 µM taxol, and 1 mM DTT) and the microtubules were pelleted by ultracentrifuging at 120K x g (28K RPM in an SW28 rotor, Beckman Coulter, Fullerton, CA) for 50 minutes at 31ºC. Following ultracentrugation, the supernatant was carefully removed to avoid contamination of the micrtobule pellet with AMP-PNP and microtubule-bound proteins (including dynein and kinesin) were released by resuspending the pellet in release buffer (PMEE, 1 mM GTP, 20 µM taxol, and 1 mM DTT, 5 mM $MgSO_4$, and 10 mM ATP) and incubating for 20 minutes at 30ºC, pipetting every 5 minutes to break up the last remaining chunks of the pellet. The dynein-free microtubules were then removed by ultracentrifuging at 210K x g (45K RPM in an SW50.1 rotor, Beckman Coulter, Fullerton, CA) for 30 minutes at 31ºC and recovering the supernatant.

Dynein was then separated from kinesin by loading the supernatant onto a 10-40% sucrose gradient (containing 1 mM DTT and 0.5 mM ATP) and ultracentrifuging at 120K x g (28K RPM in an SW28 rotor, Beckman Coulter, Fullerton, CA) for 18 hours at 4ºC. Following the spin, fractions were collected from the sucrose gradient by boring a small hole in the ethanol-wiped bottom of the tube with a 16-gauge needle and collected fractions were run on a 7.5% SDS-PAGE gel to identify dynein- and kinesin-containing fractions. To separate dynein from dynactin, the dynein-containing fractions were pooled and run through a MonoQ column (Amersham Biosciences, Pittsburgh, PA) with a 0-1.0 M KCl gradient. Peak dynein fractions, eluted from the column at 320 mM KCl, were supplemented with 0.5 mM ATP and 1 mM DTT and stored on ice.

2.3.6 Microtubule Motility Assays

The beads used for motility assays were 200 nm carboxylate polystyrene Polybeads® from Polysciences (Warrington, PA) and 200 nm NeutrAvidin®-labeled, yellow-green fluorescent polystyrene FluoSpheres® from Invitrogen (Carlsbad, CA). To prepare peptide-loaded beads, Fluospheres were incubated with an excess of biotinylated peptide for 30 minutes at room temperature and then dialyzed against 6000 volumes of PBS to remove unbound peptide.

Flow chambers were prepared by attaching a poly-L-lysine-coated coverslip to a microscope slide through two pieces of double-stick tape. Microtubules were preformed by incubating purified tubulin in final dilution buffer (FDB; 100 mM PIPES, pH 7.2, 1mM MgSO$_4$, 2 mM EGTA, 20 µM taxol, and 1 mM GTP) for 15 minutes at 37ºC and then loaded onto the coverslip surface by incubating them in the flow chamber for 30 minutes at room temperature. Unbound microtubules were removed by flowing through FDB and the surface was blocked with 5 mg/ml casein in FDB for 30 minutes at room temperature. Carboxylate-, Neutravidin-, or peptide-bound-Neutravidin-beads were mixed with purified dynein at various molar ratios in FDB plus 0.8 mM ATP and 0.2 mg/ml casein, introduced into the flow chamber, and immediately viewed by video-enhanced differential contrast microscopy using a Hamamatsu Argus-20 image processor. Images were recorded onto DVDs using a Sony RDR-GX300 DVD recorder. Recordings were later played back to identify individual beads that had bound to microtubules and to quantify movement events. Motility events included all occurrences where a bead in solution landed on a microtubule and moved linearly along the microtubule for at least

two seconds. To calculate the frequency of motility (seconds of moving/minute), the amount of movement time for all of the motility events for each condition were added together and divided by the total video time.

BART Peptide	Sequence	Binding partner	Source protein	Ref.
BART1	WCGGSGGKSSE**DKSTQT**TGREL	LC8	Rabies phosphoprotein P (RPP)	[78]
BART1C	WCGGSGGKSSE**AKSTAT**TGREL	--	2 aa mutation of RPP	[78]
BART2	WCGGDEASAT**VSKTETSQV**APA	Tctex1	Human rhodopsin C-terminal tail	[79]

Table 2-1 BART peptide sequences
The sequences for each peptide are shown along with the dynein subunit binding partner and the source protein that the peptide is derived from. The underlined sequences are directly from the source protein. The bold sequence is the putative dynein subunit binding sequence.

2.4 RESULTS

2.4.1 Design and Synthesis of BART Peptides

BART peptides sequences were selected by review of the literature to identify proteins for which (1) binding to the light chains of dynein has been shown and (2) the portion of the protein that binds to dynein has been determined. The sequences of each of the BART peptides are shown in Table 2-1 along with each peptide's protein of origin and putative binding partner. BART1C contains two point mutations from the BART1 sequence that have been shown to abolish binding to LC8 [78], allowing this peptide to be used as a control for LC8 binding. Each peptide contains a tryptophan for UV quantification of peptide concentration, a cysteine to allow for conjugation to other molecules, and a GG or GGSGG linker to extend the peptide from the conjugation site. To make biotinylated peptides, a biotin-maleimide linker was conjugated to the cysteine of each peptide. MALDI-TOF mass spectrometery of the biotinylated and unbiotinylated peptides reveals that each had the correct mass (not shown).

2.4.2 Binding between BART Peptides and Dynein Light Chains

His-tagged rat LC8 and mouse Tctex1 were expressed in and purified from *E. coli*. SDS-PAGE gel (Fig. 2-2a) revealed that each protein had the expected monomer size. Circular dichroism measurement of each protein (Fig. 2-2b) confirmed that each had the appropriate secondary structures [80, 81]. Furthermore, calibrated size exclusion chromatography (Fig. 2-2c) showed that each protein formed a dimer in solution, as expected from published structures [82, 83].
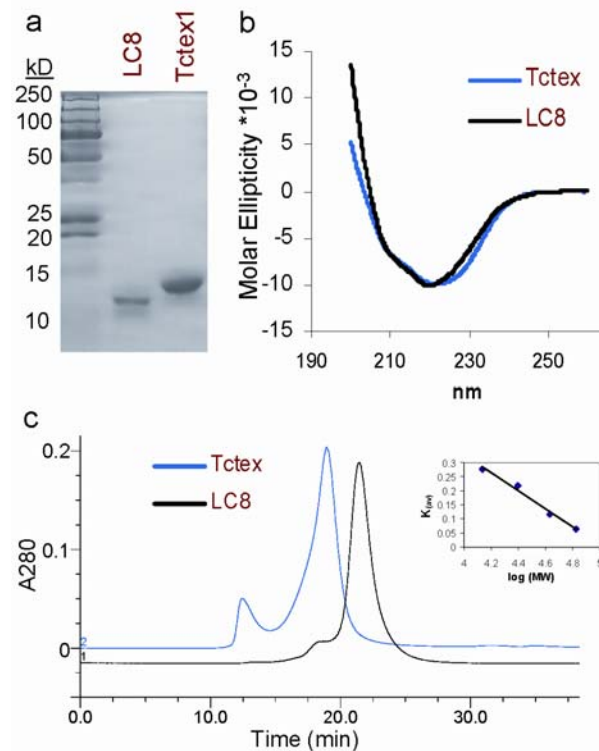
Figure 2-2 Characterization of LC8 and Tctex.
(a) SDS-PAGE gel stained with coomassie blue. (b) Circular dichroism spectrum confirming appropriate secondary structure [80, 81] (c) Calibrated size exclusion chromatography with a Supedex 75 column. The proteins used to make the size standard curve (inset) were albumin (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD), and ribonuclease A (13.7 kD). The calculated size for LC8 and Tctex were 22 kD and 31 kD, respectively, indicating that both most likely form dimers in solution.
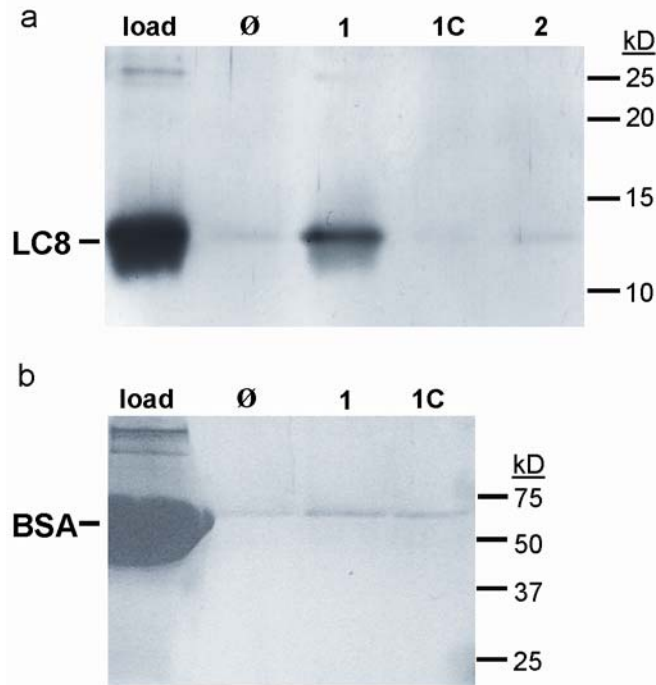
Figure 2-3 Binding between BART peptides and LC8.
BART peptide pulldown assay. Streptavidin-agarose columns were loaded with either (1) BART1, (1C) BART1C, (2) BART2, or (Ø) no peptide. LC8 (a) or bovine serum albumin (BSA, b) that were run through the column and any protein that were retained were eluted with a high salt, high pH buffer and run in an SDS-PAGE gel stained with Silver Stain.

A streptavidin pulldown assay was used to evaluate the binding of BART peptides to the dynein light chains. LC8, Tctex1, or BSA were run separately through a streptavidin-agarose spin column pre-loaded with one of the biotinylated BART peptides (or without peptide) and bound protein was eluted with a high-salt, high-pH buffer. Protein in the elution buffer was then detected by SDS-PAGE (Fig. 2-3). LC8 was retained on the BART1 column but not on the unloaded (streptavidin-only), BART1C, or BART2 column (Fig. 2-3a) indicating that indeed there is an interaction between LC8 and BART1 that is inhibited by the two amino acid mutation that is present in BART1C. This is the first time to our knowledge that this sequence from RPP alone (not as part of the full RPP protein) was show to bind to LC8 independent of the full length RPP. BSA

(Fig. 23b) and Tctex1 (not shown) were not retained on any of the peptide-loaded or unloaded columns further confirming the specificity of the LC8-BART1 interaction.

It is important to note that Tctex1 was not retained on the BART2 column, despite the fact that the BART2 peptide was designed to bind to Tctex1. It is possible that the interaction between BART2 and Tctex1 is very weak or that BART2 does not contain enough of the rhodopsin sequence to allow for binding to Tctex1 (see discussion).

2.4.3 Dynein-dependent *in vitro* Motility of BART-Peptide Beads

To investigate the ability of dynein to carry cargo that is bound to BART1, we set up a reconstituted *in vitro* microtubule motility assay with active dynein complex purified from fresh cow brain. Dynein was purified by a multi-step process which involved a variety of biochemical separation techniques to remove non-dynein proteins from cleared brain homogenates. First, proteins that do not bind to microtubules were removed by a microtubule affinity step in which dynein was bound tightly by AMP-PNP to pelleted microtubules and eluted with ATP. Second, dynein was separated from other microtubule-binding proteins, including kinesin, by a 16-hour ultracentrifugation through a sucrose gradient. Lastly, dynein was separated from the dynactin complex through a MonoQ anion-exchange column. Purified dynein complex components, including dynein heavy chains, intermediate chains, and light intermediate chains can be visualized on a Silver Stained SDS-PAGE gel (Fig. 2-4). The resulting dynein complex remained active for not more than three days.
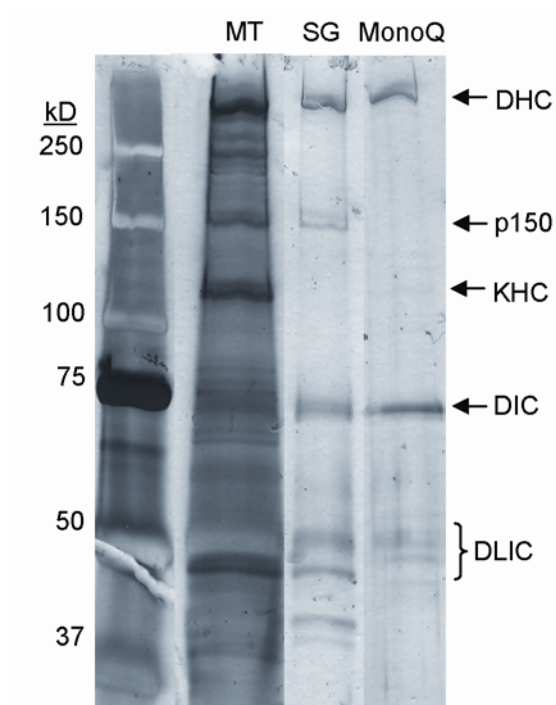
Figure 2-4 Dynein purification.
Dynein was purified from cow brain as described in Methods. Fractions from the following steps were run on an SDS-PAGE gel and stained with Silver Stain: MT, microtubule affinity; SG, sucrose gradient; MonoQ, MonoQ anion-exchange column. Molecular motor subunits are indicated with arrows or bracket: DHC, dynein heavy chain; p150, dynactin p150; KHC, kinesin heavy chain; DIC, dynein intermediate chain; DLIC, dynein light intermediate chains.

The cargo used for the microtubule motility assays were 200 nm streptavidin-coated polystyrene beads that were coated with biotinylated BART1 or biotinylated BART1C and could be visualized with a differential interference contrast (DIC) microscope. Each set of beads were mixed with dynein at a range of molar ratios along with ATP to fuel the motor and casein to prevent nonspecific binding to the glass surface, and added to a flow chamber containing preformed immobilized microtubules. Carboxylated beads (positive control) bound to dynein at all ratios tested and were robustly carried along microtubules by dynein while streptavidin beads (without peptides, negative control) were not observed to bind to or travel along microtubules at all (data not shown).
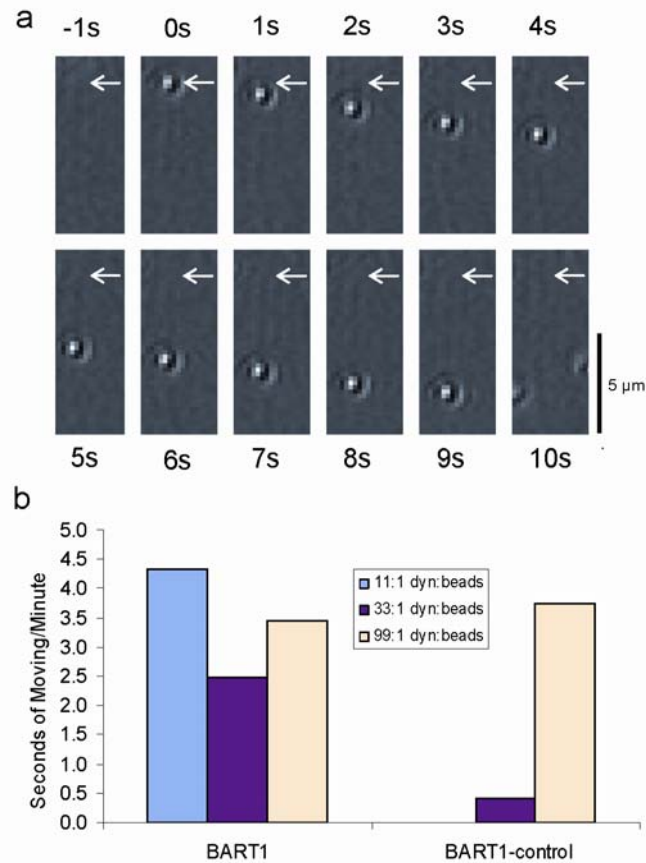
Fig. 5. Dynein motility assays

Figure 2-5 Dynein motility assays.
Peptide-coated beads were tested for their ability to be carried along microtubules by dynein in an *in vitro* flow chamber (a) Example of a BART1-coated bead being carried along a microtubule by dynein. Each frame represents a single frame of a time-lapse video, spaced one second apart. The arrow indicates the position of the bead when it bound to the microtubule at time 0 seconds. After 9 seconds, the bead released from the microtubule and diffused away. (b) Analysis of the total amount of bead movement under each condition. 20-30 minutes of video were collected and analyzed for each condition.

BART1-bound beads at a dynein:bead ratio of 11:1 could be seen both in suspension above the microtubules and moving actively along microtubules in a dynein-like manner (Fig. 2-5a) while BART1C-bound beads stayed only in suspension above the microtubules (not shown). These data suggest that dynein was able to interact with the BART1 peptide while retaining its active transport function. At higher dynein:bead ratios, some movement of BART1C-bound beads was also observed, indicating that there

may be some non-specific interaction between BART1C and dynein which is not observed at the lower dynin:bead ratio at which, only BART1-beads are motile. These data, summarized in Figure 2-5b, provides the first evidence that a peptide could be used to target novel cargo to the native dynein complex for transport along microtubules.

2.5 DISCUSSION

We have demonstrated here the design of a BART peptide that can link cargo to a dynein light chain and mediate the transport of that cargo along microtubules in a reconstituted system. The dynein- and BART-dependent transport we observe is not as robust as naturally occurring intracellular transport and so would most likely require further improvements to be used as an intracellular tool. Nonetheless, this approach represents the first step towards a synthetic viral-mimic strategy to delivery DNA to the cell nucleus, which has the potential to greatly improve non-viral gene therapy.

There are multiple subunits of dynein that have been suggested to be responsible for cargo-binding including dynein light chains, dynein light intermediate chains, and another protein complex called dynactin that interacts closely with dynein. We chose to focus on two of the light chains of dynein, LC8 and Tctex, because binding to these proteins has been well characterized, including the structures of these proteins with bound peptide [82-86]. Based on structural analysis, it appears that peptides containing the LC8-binding consensus sequence bind to the LC8 dimer in the same two identical binding pockets as the dynein intermediate chain (DIC) [87]. The binding between LC8 and DIC are essential for LC8's incorporation into the dynein complex, which could raise the question of how LC8 could bind to both its cargo and dynein at the same time.

However, the actual structure of the intact dynein complex is not known and so it remains possible that LC8 could bind to DIC through one of its binding pockets and to its cargo through the other.

The sequences for BART peptides (Table 2-1) were chosen based on evidence for their binding to a dynein light chain, for their importance for retrograde transport and the availability of crystal structures showing how a similar peptide binds to its respective light chain. The LC8-binding sequence, BART1, was derived from a portion of the Rabies phosphoprotein P (RPP) that contains an LC8 binding consensus sequence, (K/R)XTQT [88]. This portion of RPP was shown by co-immunoprecipitation, a two-hybrid screen, and confocal co-localization to interact with LC8 [78, 89, 90] and deletion of this portion of RPP has been shown to attenuate the infectivity of the virus [70, 91]. Using a streptavidin pull down assay, we have shown that the BART1 peptide binds to LC8 but not to Tctex or BSA (Fig. 2-3). Furthermore, BART1C (Table 2-1), which contains two mutations in the RPP sequence of BART1 that have been shown to knockout binding to LC8 [78], did indeed not bind to LC8 (Fig. 2-3a). These results indicate that BART1 binds specifically to LC8 in the same manner as the full RPP protein and thus could be used as linker to dynein if the peptide can bind to LC8 within the intact dynein complex.

The putative Tctex-binding peptide, BART2, was derived from the c-terminal tail of human rhodopsin. This portion of rhodopsin has been shown to bind to Tctex by co-immunprecipitation, a two-hydrid screen, and immunoelectron microscopy co-

localization [79]. Naturally occurring mutations within this region of rhodopsin in patients with retinitis pigmentosa have been shown to prevent proper intracellular localization of rhodopsin and to interfere with the binding of rhodopsin to Tctex [79]. Furthermore, rhodopsin-carrying vesicles were shown to travel along microtubules in a dynein- and Tctex-dependent manner [79]. Despite these previous results, the BART2 peptide failed to bind to Tctex in our streptavidin pull down assay and BART2-bound beads failed to be transported along microtubules by dynein (data not shown). One possible reason for this discrepancy is that BART2 contains only the terminal 18 residues of rhodopsin and not the entire 39-residue c-terminal tail. Although the previous study has shown that the terminal 9 residues of rhodopsin are critical for Tctex-binding, all of those binding studies were done with a triple-repeat of the full c-terminal tail [79]. It is possible then that a next-generation BART peptide containing the full c-terminal tail of rhodopsin could bind to Tctex, though efficient binding might actually require the triple repeat of the full c-terminal tail (T.Y. Yeh, Weill Medical College of Cornell University, personal communication). Nonetheless, Tctex should be considered as a target cargo-binding subunit of dynein because unlike LC8, Tctex appears to bind to its cargo though a site unique from its dynein-intermediate-chain-binding site [86].

To evaluate the interaction of BART peptides with dynein, we chose to use an *in vitro* reconstituted microtubule motility assay because (1) this assay will detect only functional interactions between BART and dynein, and (2) the single-molecule nature of this assay would allow us to identify rare events. Similar assays have been used to characterize the motility of dynein itself [92, 93], of pigment granules isolated from

Xenopus melanophores [94], and of endosomes isolated from HeLa cells [95]. In the present study, polystyrene beads were coated with one of the BART peptides in Table 2-1 and exposed to purified cytoplasmic dynein in a flow chamber containing immobilized microtubules. Analysis of video recordings of the beads in the chamber (Fig. 2-5) revealed multiple dynein-based motility events with LC8-binding BART1 beads even at the lowest dynein:bead ratio tested (~10:1) while dynein-based motility of the control BART1C peptide-beads (Fig. 2-5) requires a very high dynein:bead ratio (~100:1) at which nonspecific binding is likely to occur. Furthermore, dynein-based motility of unloaded (streptavidin-only) beads or BART2 beads did not occur even at this high ratio (not shown). These data suggest that there is a specific interaction between BART1 and dynein. Motility experiments in the presence of excess LC8 could be performed to confirm that BART1-dynein binding occurs through LC8.

Further investigation is clearly required to determine if targeting novel cargo to dynein is a feasible means of improving retrograde transport *in vivo*. While the dynein-based motility events of BART1 beads were easily observable in our reconstituted system, these were still rare events (less than one per minute), indicating that there remains a fair amount of optimization of the BART-peptide system if it is to be used inside of cells. Indeed, previous efforts to use LC8 binding sequences to target novel cargos to dynein inside cells have had mixed results: one study indicates that proteins containing the LC8-binding sequence of adenovirus-associated BS69 protein failed to bind to dynein inside cells [9] while another study has shown that proteins containing the LC8-binding domain of RPP (LC8-BD), which is the same as BART1, can interact with microtubules and

improve nuclear localization signal (NLS)-dependent nuclear accumulation [10]. The results presented here are an encouraging first-step towards a system to target novel cargo to the intracellular motility system and it is likely that as our understanding of dynein structure and function improves, further strategies for linking cargo to dynein will become available.

2.6 ACKNOWLEDGEMENTS

CHAPTER 3: Quantification of Plasmid DNA Copies in the Nucleus after Lipoplex and Polyplex Transfection

3.1 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 Lipofectamine™ 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.

3.2 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 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 [96, 97], targeting agents for internalization into specific cell types [98-101], pH-responsive agents that can disrupt endosomes to allow release of DNA into the cytoplasm [66, 96, 99], motor-protein-binding peptides that can allow for active transport of DNA towards the nucleus [9, 73], and nuclear localization signal peptides that may trigger import of associated cargo into the nucleus [102].

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 [103]. 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 [25]. 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 [104] 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.

3.3 METHODS

3.3.1 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).

3.3.2 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 37ºC, 5% CO_2. 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 37ºC, 5% CO_2 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 37ºC, 5% CO_2 for 3 hours. Following removal of polyplexes or lipoplexes from the cells and an additional 21 hour incubation at 37ºC, 5% CO_2 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.
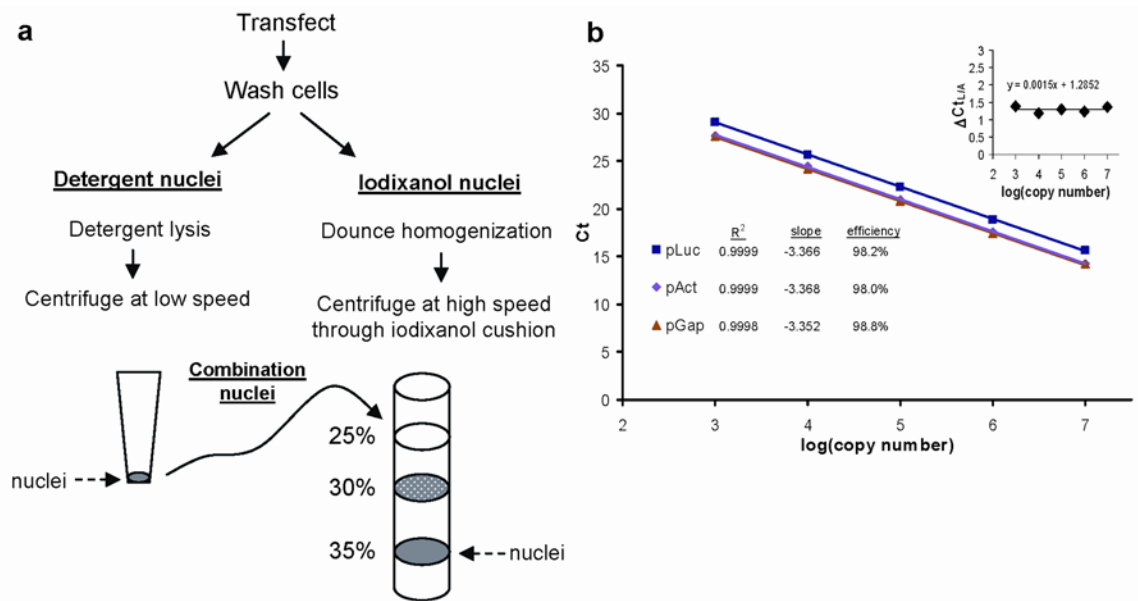
Figure 3-1 Nuclear isolation and quantitative PCR.
(a) Diagram of nuclear isolation methods used (see methods). (b) qPCR standard curves used for relative quantification of plasmids/nucleus. The threshold cycle (Ct) for various concentrations of plasmids containing either the luciferase sequence (pLuc), the genomic actin sequence (pAct) or the genomic gapdh sequence (pGap) were measured to calibrate the qPCR assay. The R^2 value and slope of the linear regression lines and the calculated PCR efficiencies ($E = 10^{(1/slope)} - 1$) are shown. The difference between the Ct of pLuc and the Ct of pAct ($\Delta Ct_{L/A}$) 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.

3.3.3 Isolation of Nuclei from Mammalian Cells

Nuclei were isolated from A549 or B16F10 cells by three different methods (Fig. 3-1a). (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 $MgCl_2$) 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 x 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 ℃, pelleted by centrifuging at 1400 x g for 5 minutes, washed twice with PBS and resuspended in 1 ml nuclei buffer (50 mM PIPES, 0.5M DTT, 0.5 M $MgCl_2$, 1 M 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 Na_2HPO_4, 1 mM $MgCl_2$, 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 x 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 20ºC.

3.3.4 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 37ºC, 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 LaserSharpe 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.

3.3.5 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 4ºC 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 37ºC with shaking followed by a 30 minute incubation at 65ºC to inactivate the enzyme.

3.3.6 DNA Extraction

Isolated nuclei were lysed by incubating them in 10% SDS with 20 mg/ml proteinase K and 500 µg/ml DNAse-free RNAse for 4-5 hours at 50ºC. 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 -70ºC or overnight at -20ºC, washed with 70% ethanol, and stored in TE buffer (Qiagen, Valencia, CA) at 4ºC.

3.3.7 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 Integraded DNA Technologies (Coralville, IA) and have the following sequences- forward primer (LucF): GTACACGTTCGTCACATCTC, 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. 3-1b). The difference in Ct values between luciferase and actin (Fig. 3-1b, 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 $L/A = 2^{-\Delta\Delta Ct}$ where the $\Delta\Delta Ct = \Delta Ct_{samples} - \Delta Ct_{calibrators}$ [105]. 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.

3.3.8 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.

3.4 RESULTS

3.4.1 Isolation of Nuclei with Minimal Extranuclear DNA

To prepare nuclei with minimal extranuclear plasmid DNA, we compared three nuclear isolation methods (Fig. 3-1a). For the first method ("detergent nuclei") [106], 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") [104], 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. 3-1b, 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

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combination nuclei. (Fig. 3-2a). 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 polyplexes (Fig. 3-2c) or lipoplexes (not shown) were seen on the outside of iodixanol nuclei, which are mostly dispersed (0-5% of nuclei in aggregates) (Fig. 3-2c). Nuclear
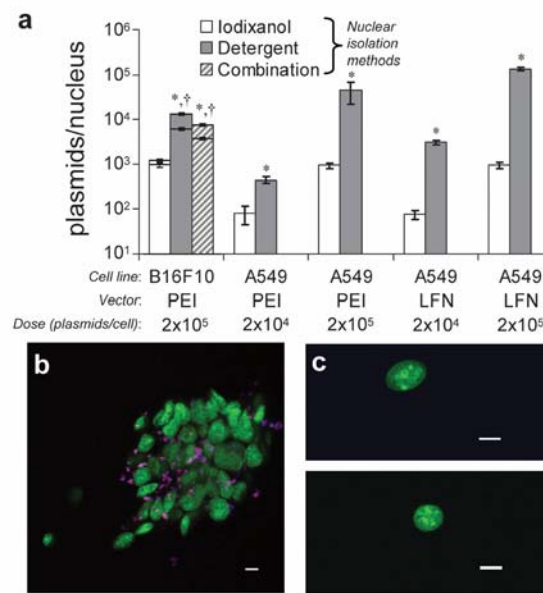
Figure 3-2 Comparison of nuclear isolation methods.
(a) Detected plasmids/nucleus using the PCR assay for nuclear DNA extracted from nuclei isolated from transfected cells using the indicated methods (Fig. 3-1a). *Indicates $P<0.05$ (Student's t-test) in comparison to iodixanol nuclei from same transfection 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 μm.

extranuclear polyplexes (Fig. 3-2b) or lipoplexes (not shown). On the contrary, no visible 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.

3.4.2 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. 3-3a) 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 βgal plasmid, each of which could potentially compete with electrostatic interactions between the DNA and the nucleus, had limited or no success (Fig. 3-3b). 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. 3-3b). 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.
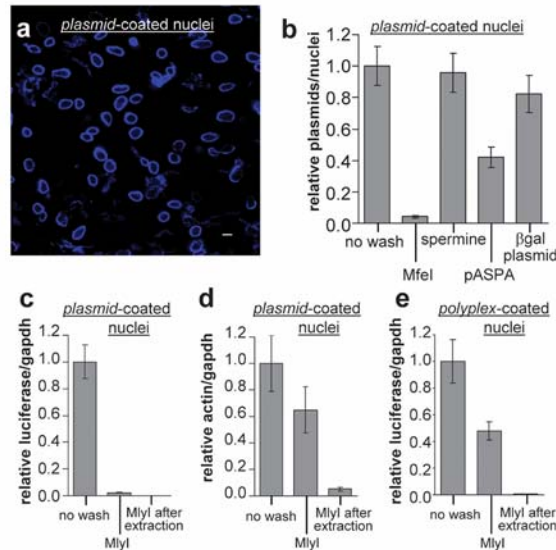
Figure 3-3 Testing wash conditions to remove extranuclear DNA from plasmid- and polyplex-coated nuclei.
(a) Confocal microscope image of nuclei isolated from untransfected B16F10 cells by the detergent method, incubated with Cy5-pLuc for 1 hour at 4ºC and washed with lysis buffer. Scale bar = 10 µm. (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.

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. 3-3c), as was the case with MfeI (Fig. 3-3b). However, 35-40% of the actin sequence is also digested (Fig. 3-3d), 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. 3-3e), indicating that the PEI can protect the plasmid from digestion as has been previously reported [107]. When total DNA extracted from the polyplex-coated nuclei are incubated with MlyI, greater than 99% of the luciferase sequence is cut (Fig. 3-3e), 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 Figure 3-2a. 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. 3-2a), 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.

3.4.3 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 ($\sim10^7$ 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. 3-4a,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. 3-4b,d).
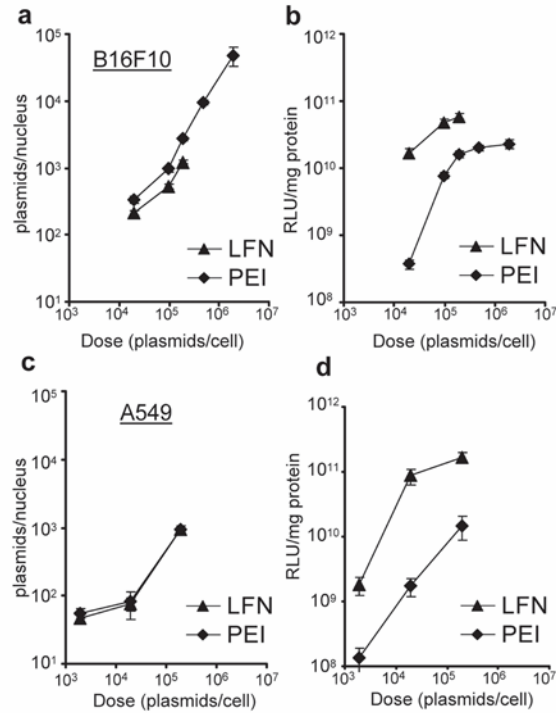
Figure 3-4 Plasmids/nucleus and luciferase expression dose response of PEI polyplexes and LFN lipoplexes.
B16F10 (a-b) or A549 (c-d) cells were transfected with PEI/pLuc polyplexes or LFN/pLuc lipoplexes at the indicated doses. (a,c) Detected plasmids/nucleus in nuclei isolated by the iodixanol method. (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.

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. 3-4a,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. 3-4). 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 $2x10^5$ plasmids/cell to $5x10^5$ and $2x10^6$ plasmids/cell is relatively small (1.3- and 1.4-fold, respectively) (Fig. 3-4b) while the corresponding increase in plasmids/nucleus is much larger (3.5- and 17.8-fold, respectively) (Fig. 3-4a). 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. 3-4c,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.

3.4.4 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. 3-5b,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.
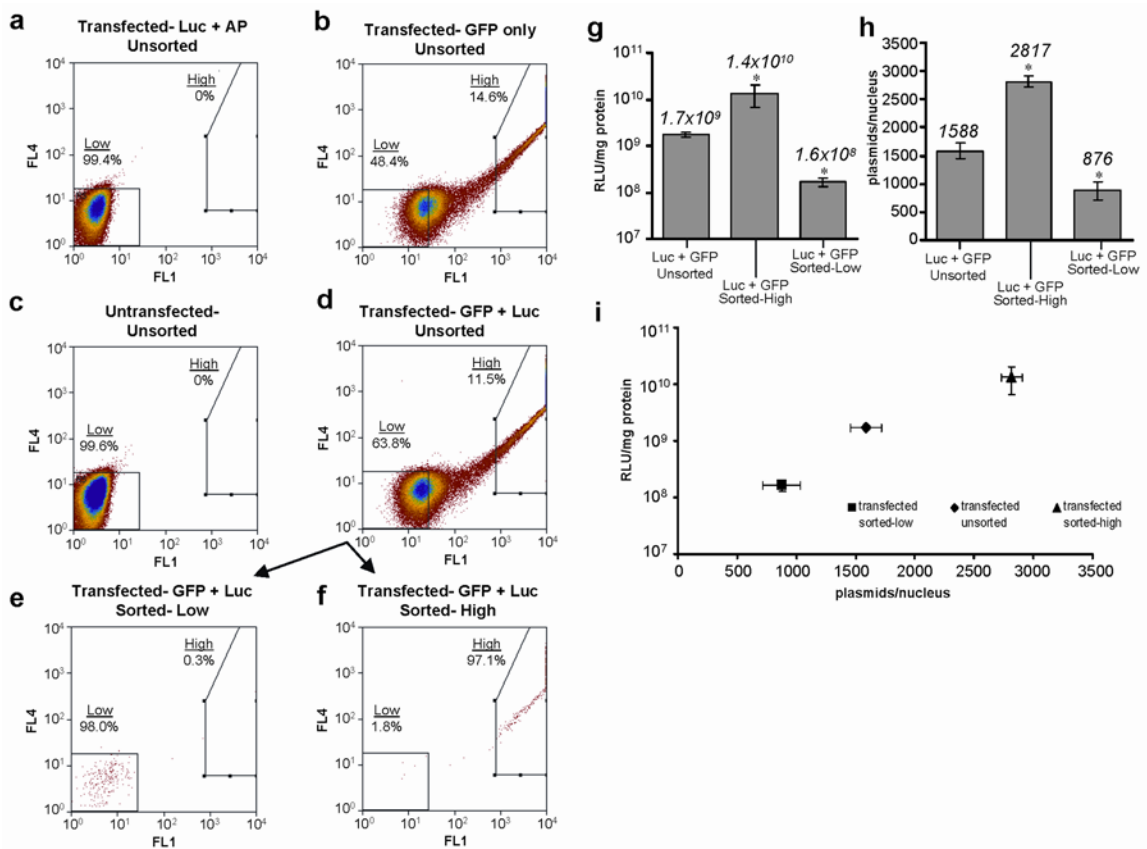
Figure 3-5 Measuring plasmids/nucleus in subpopulations of high- and low-expressing cells.
B16F10 cells were co-transfected with 10^5 copies/cell each of PEI/pLuc and PEI/pAP (secreted alkaline phosphatase) polyplexes (a), PEI/pGFP only (b), no polyplexes (c), or PEI/pLuc and PEI/pGFP polyplexes (d). 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.

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. 3-5d). 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. 3-5a) is indistinguishable from a similar plot for untransfected cells (Fig. 3-5c). Likewise, a plot of FL4 vs, FL1 for cells transfected with only the GFP plasmid (Fig. 3-5b) 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. 3-5d). 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. 3-5d) were sorted for GFP expression after 24 hours into relatively high- (Fig. 3-5e) and low-expressing (Fig. 3-5f) cells. Luciferase expression (Fig. 3-5g) was correlated with plasmids/nucleus (Fig. 3-5h) 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. 3-5i). 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.

3.5 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 [106, 108-111] 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. 3-2b). Furthermore, plasma-membrane lipid rafts have been detected in nuclei isolated from detergent-lysed cells [112]. 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. 3-3, [106, 109]). In this study we have investigated the use of mechanical lysing of cells followed by iodixanol density gradient ultracentrifugation [104]. This "iodixanol method" allows for the preparation of nuclei with minimal extranuclear DNA (Fig. 3-2c) 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. 3-2a). 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 [106, 110, 111]. In this paper, we extend this method with the use of the $2^{\Delta\Delta Ct}$ relative quantification method [105] 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 [113] as well as the copy number of chromosomal genes in mammalian cells [114, 115]. 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. 3-1b), (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. 3-4). Previous studies [106, 111] 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 [109] or Southern Blot of DNA extracted from isolated nuclei [106, 110] measure plasmids/nucleus in this same range, a recent investigation of nuclear delivery by LFN complexes in A549 cells by Hama et al [116, 117] using fluorescently labeled plasmid DNA and a confocal microscopy technique measured greater than $4x10^4$ 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. 3-4a, c) despite the fact that the LFN transfection resulting in significantly greater expression than PEI- typically 10-fold higher (Fig. 3-4b, 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 [26]. Though it is not clear to what extent DNA release occurs before or after delivery of the DNA into the nucleus [25, 118, 119], 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 [8]. 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 [6, 120]. 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 [121] and lipoplexes in the cytoplasm may fuse directly with the nuclear membrane to release their contents to the interior of the nucleus [122]. 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. 3-5a) 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. [123] 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. 3-5a). We then isolated the nuclei from each sub-population using the iodixanol method and quantified plasmids per nucleus in each using quantitative PCR (Fig. 3-5b). 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. 3-5c). 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 [124]. 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 [21, 22] 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 [125] 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 [126]. 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 [111] 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 [13] showed that thymidine kinase (TK) activity could be detected in a TK-deficient mouse fibroblast cell line following the nuclear injection of as a 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 [127]. 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 [128]. Such studies will further enhance our understanding of the nuclear events that occur during transfection and could contribute to the design of novel vectors.

3.6 ACKNOWLEDGEMENTS

CHAPTER 4: High Throughput Screen for Small Molecule Enhancers of Transfection

4.1 ABSTRACT

The objective of non-viral gene delivery is to use relatively simple molecules to copy the processes and molecular strategies employed by viruses in the infection process. However, non-viral delivery systems have been unable to achieve the gene transfer efficiency of viruses. We have developed a high throughput transfection assay to screen a large chemical library of low molecular weight compounds to identify molecules that enhance gene transfer mediated by non-viral vectors. The assay was validated using polyethylenimine (PEI) transfection of a green fluorescent protein (GFP) plasmid into B16F10 mouse melanoma cells but could be adapted for use with other vectors, reporter genes, and cell lines. Conditions were determined that minimize the steps required to transfect cells with a baseline dose of PEI/pGFP polyplexes and to measure the expression of GFP. The Z-factor of the assay was calculated to be at least 0.36, indicating the assay is capable of identifying transfection enhancers. Pilot runs of the assay revealed a normally distributed effect of the test compounds with less than 0.5% of compounds resulting in fluorescence values greater than three standard deviations above the mean value. Of the 38 positive hits detected in a small-scale (1,600 compounds) and a medium-scale (6,080 compounds) run of the assay, all were identified as false positives caused by intrinsic fluorescence of the compounds themselves, as observed by

fluorescent microscopy. This simple secondary screening step could quickly be performed in less than one day for all of the positives hits identified in a full-scale screen of 100,000 compounds. The robust assay described in this study could help identify new molecules that could greatly improve the efficiency of non-viral vectors and could aid in our understanding of rate-limiting steps in the transfection process.

4.2 INTRODUCTION

The delivery of gene medicines by synthetic carriers is a complicated process that requires plasmid DNA to circumvent numerous barriers to gene expression. These barriers include: 1) the packaging of DNA (forming the complex) 2) delivery to the target cell 3) cell internalization via endocytoses 4) the unpackaging of DNA from the vector, 5) DNA escape from the endosome, 6) DNA transport through the cytoplasm, 7) nuclear entry, 8) gene expression, and 9) plasmid retention. Viruses have evolved to overcome these barriers; synthetic vectors must be engineered to do so. Efforts to rationally design better vectors are underway. Novel cationic carrier compounds, such as cationic lipids and polycationic polymers have been the most successful as synthetic vectors [129]. These compounds bind to DNA via electrostatic interactions and compact DNA as well as protect it from nuclease degradation. The net positive complexes that are generated can be advantageous for binding to negatively charged cell surfaces. In addition, pH-sensitive lipid vectors that mediate efficient cytoplasm entry after internalization into cells have been synthesized [130] and nuclear targeting proteins have been added to help navigate the DNA through the cytoplasm (after endosomal release) and into the nucleus [131]. These components are usually chemically incorporated into the complex.

Low molecular weight compounds that enhance transfection include: chloroquine [132], glycerol [133], DNAse inhibitors [134], progesterone [135], sodium butyrate [136], and colchicine [137]. These transfection enhancers were identified through a knowledge-based approach: that is, they were originally studied due to their ability to modulate cellular compartments or processes that were suspected to be involved during the entry of vectors into cells. They were not discovered in a manner that involved an extensive or systematic search of drug-like compounds.

Given these specific examples, it is reasonable to believe that other chemicals exist that can improve known steps involved in gene transfer. Furthermore, a large scale, unbiased search might uncover new classes of low molecular weight molecules to enhance gene transfer. These putative undiscovered chemical transfection enhancers will possibly exhibit greater potency than known activators. The criteria for selecting such molecules include low cytotoxicity and a beneficial effect on one or more steps in gene transfer. A safe enhancer of gene transfection could be incorporated into the delivery vector or co-delivered with the delivery vector. In the lung an enhancing molecule could be administered via inhalation in a coordinated manner, either before, during or after administration of the synthetic vector. The identification of novel enhancers could also provide insight into known or unknown mechanisms of gene transfer.

Our approach to identifying new classes of transfection enhancers involves a cell-based high-throughput screen of diverse low molecular weight drug-like compound libraries. Since the assay is cell-based, the screen is designed to identify compounds that

enhance transfection by *in vitro* effects. Similar screens are widespread in the pharmaceutical industry and are used in academic settings to identify small molecule activators of wild type human cystic fibrosis transmembrane conductance regulator protein (CFTR) [138], small molecule inhibitors of the Hypoxia-inducible factor 1 (HIF-1) pathway [139], and small molecule inhibitors of herpes simplex virus type 1 alkaline nuclease [140]. Moderate-throughput screens have also been used to identify the optimal cationic lipids to be used with specific cells as synthetic vectors for gene transfection [141, 142]. Thus the concept is well validated, applying it in a logical fashion to the discovery of chemical adjuvants to improve gene transfer is the raison d'etre of this study.

4.3 METHODS
4.3.1 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. pCMV-GFP (pGFP, 3831 bp) was a generous gift from Valentis (Burlingame, CA). The compound libraries to be screened were purchased from ChemDiv (San Diego, CA) and ChemBridge Corp. (San Diego, CA) and consist of 100,000 structural diverse drug-like molecules.

4.3.2 Cell Culture and Transfection

B16F10 mouse melanoma cells were seeded in 96-well black-walled plates

(Costar, Corning, NY, USA) at 10^5 cells/well in media (MEM Eagle's with Earle's BSS containing 1% Sodium pyruvate, 1% non-essential amino acids) with 10% fetal bovine serum and incubated for 24 hours at 37ºC, 5% CO_2. Cell media was changed to either fresh media with 10% serum or Advanced MEM media (GIBCO, Grand Island, NY) with 1-5% serum. Polyplexes were prepared by combining PEI and the GFP plasmid at a 1.8:1 (+/-) ratio in serum-free media and incubating at room temperature for 30 minutes. Polyplexes were then added directly to media in the wells to produce desired concentration and incubated at 37ºC, 5% CO_2 for the specified time. Cells incubated with polyplexes for 4 hours were then changed to fresh media with 10% serum for the remainder of the time. Following the transfection, GFP fluorescence was measured with a FLUOstar Optima fluorescence plate readers (BMG Labtechnologies, Durham, NC).

4.3.3 High-Throughput Screen

Screening was performed with a customized screening system (Beckman Coulter, Inc., Indianapolis, IN) consisting of the SAGIAN Core system integrated with SAMI software, and equipped with an ORCA arm for labware transport, a 96-channel head Biomek FX, plate rack, CO_2 incubator, plate washer, delidding station, and two FLUOstar Optima fluorescence plate readers (BMG Labtechnologies, Durham, NC). Transfection conditions were the same as described above with one exception: a mixture of four compounds (final concentration, 2.5 µM each) from the compound library were added to the cells 30 minutes to 2 hours prior to addition of polyplexes. A diagram of the layout of each 96-well plate is shown in Figure 4-4.

The screen proceeded as follows: (1) Compound addition: (a) Cell plate containing cells incubated overnight transported from incubator to liquid handling station, (b) Compound plate containing stocks of library compounds transported from plate rack to liquid handling station, (c) Compounds aspirated from compound plate and added to appropriate wells of cell plate, (d) Cell plate returned to incubator and compound plate returned to plate rack. (2) Complex addition (following addition of compounds to all plates): (a) Reservoir containing prepared polyplex manually placed in liquid handling station, (b) Cell plate transported from incubator to liquid handling station, (c) Polyplexes aspirated from reservoir and added to each well of cell plate to the appropriate final concentration, (d) Cell plate returned to incubator. (3) Fluorescence measurement (following 24 hour incubation): (a) Cell plate transported from incubator to plate washer, (b) Cell plate washed with PBS, (c) Cell plate transported from plate washer to fluorescence plate reader, (d) Fluorescence read with plate reader (100 flashes per well) using a 470/40 excitation filter and a 515/30 emission filter (Chroma, Rockingham, VT) and data recorded by computer.

4.3.4 Microscopy

Positive hit wells from high throughput screen were examined visually by epifluorescence microscopy on a Nikon Eclipse TS1000 fluorescence microscope (Nikon Instruments, Melville, NY) using a 20x objective and images were recorded using SPOT version 3.5 (Diagnostic Instruments, Kanagawa, Japan).

4.4 RESULTS

4.4.1 Assay Development

We have established a screen using enhanced green fluorescent protein GFP as the reporter plasmid (pGFP) to screen a library of compounds for their ability to enhance transfection of B16F10 cells by the cationic polymer polyethyleneimine (PEI). The screen is based on a 96-well plate transfection procedure modified for use in a high-throughput system. Green Fluorescent Protein plasmid (pGFP) is used as the reporter gene because quantification of GFP levels requires neither substrates nor cofactors due to the intrinsically fluorescent nature of the protein which is measured with a standard plate-reading fluorometer. Previous GFP based high throughput assays have been able to measure statistically relevant levels of GFP expression compared to control [142]. Preliminary experiments were performed to 1) optimize the assay to minimize the steps required for high throughput screening, 2) determine the appropriate dose such that without an enhancing molecule present, a baseline gene expression level can be measured, and 3) determine the sensitivity of the assay to the time between complex formation and transfection.

Most transfection protocols call for complexes (eg. PEI/pDNA polyplexes) to be prepared in serum-free medium and incubated with cells for 3-4 hours in serum-free medium, after which the cells are washed and the media is changed back to media with 10% serum until the reporter gene is measured. For the high-throughput screen, we sought to eliminate this media changing step. This requires that the cells are able to tolerate an overnight incubation with the complexes in serum-free or low-serum media. Minimizing the amount of serum in the media is required to minimize the inhibition of

binding of the complexes and/or test compounds to the cells by serum components. Fortunately, B16F10 cells incubated for 24 hours with PEI/pGFP complexes in media with 10% serum (without any change of media) have similar or greater expression than cells incubated for four hours with PEI/pGFP complexes in serum-free media and then changed to media with serum for an additional 20 hours (data not shown). Furthermore, GFP expression following a 24 hour transfection increases with decreasing amounts of serum in the media at least as low as 1% serum, (Fig. 4-1) using Advanced MEM from GIBCO, which is optimized for low-serum applications. Cell toxicity is observed if the cells are left in completely serum-free media for 24 hours. Therefore all transfections for the screen were performed in Advanced MEM media with 1% serum.
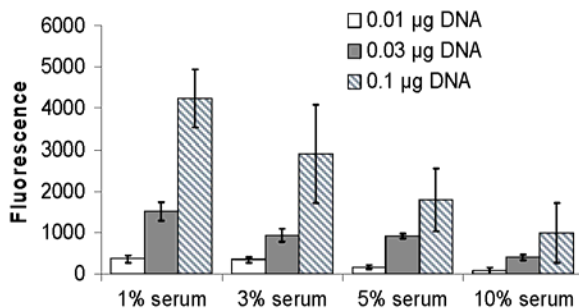
Figure 4-1 Sensitivity of a PEI/pGFP transfection dose response curve to low-serum media. B16F10 cells were grown to 70-80% confluency in a 96-well plate and incubated for 24 hours with the indicated dose of PEI/pGFP polyplexes prepared 30 minutes earlier in the indicated percentage of serum. The fluorescence in each well was then measured with a fluorescence plate reader and subtracted from the fluorescence of untreated wells.

Baseline transfection can be achieved by using a suboptimal DNA dose with an optimal charge ratio between vector and plasmid. Cells transfected with the optimal dose of DNA can then be used as positive control, in the absence of other known enhancers. In the presence of enhancing compounds, expression would be significantly increased over baseline. To determine the DNA dose to use for baseline and positive control

expression and the optimal transfection duration, B16F10 cells were transfected with increasing amounts of DNA and the amount of GFP expression was determined over time from 16 hours to 40.5 hours (Fig. 4-2). As expected, gene expression increased over time, as did the separation between expression of different doses. However, at 36 and 40.5 hours many of the cells had died (data not shown) and thus 24 hours was chosen as the optimal transfection time. A dose of 0.1 µg DNA per well (Fig. 4-6b) was chosen as the positive control because higher doses (greater than or equal to 0.3 µg) resulted in cell toxicity. A dose of 0.01 µg DNA per well (Fig. 4-6c) was chosen as the baseline dose because of the large separation from the positive control.
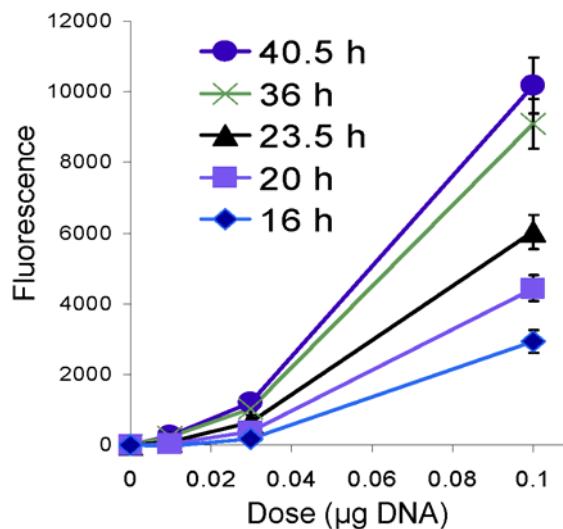
Figure 4-2 Dose response of PEI/pGFP transfection at various durations.
B16F10 cells were grown to 70-80% confluency in a 96-well plate and incubated for the indicated number of hours with the indicated dose of PEI/pGFP polyplexes prepared 30 minutes earlier in 1% serum. The fluorescence in each well was then measured with a fluorescence plate reader and subtracted from the fluorescence of untreated wells.
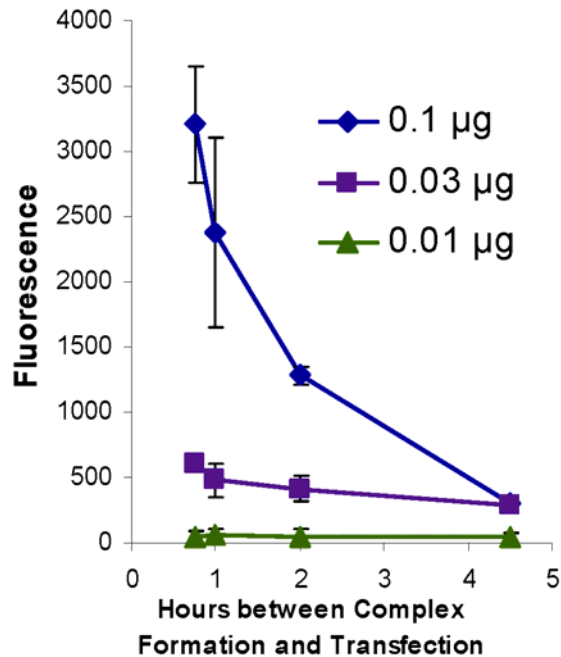
Figure 4-3 Dose response of PEI/pGFP transfection following different times between complex formation and transfection.
B16F10 cells were grown to 70-80% confluency in a 96-well plate and incubated for 24 hours with the indicated dose of PEI/pGFP polyplexes prepared in 1% serum at the indicated times before transfection. The fluorescence in each well was then measured with a fluorescence plate reader and subtracted from the fluorescence of untreated wells.

To determine the sensitivity of the assay to the time between complex formation and transfection, PEI/pGFP complexes were prepared and added to cells after a specified amount of time. GFP expression was then measured 24 hours after the addition of the complexes (Fig. 4-3). Based on these results, the time between complex formation and transfection has a large effect on the high dose (0.1 µg DNA) positive control, indicating that the complexes may settle or become unstable after more than one or two hours. This effect can be partly accounted for by including positive control wells on every plate and adding all complexes (for test wells and controls wells) at approximately the same time on each plate. In addition, complexes should be used less than two hours after formation.
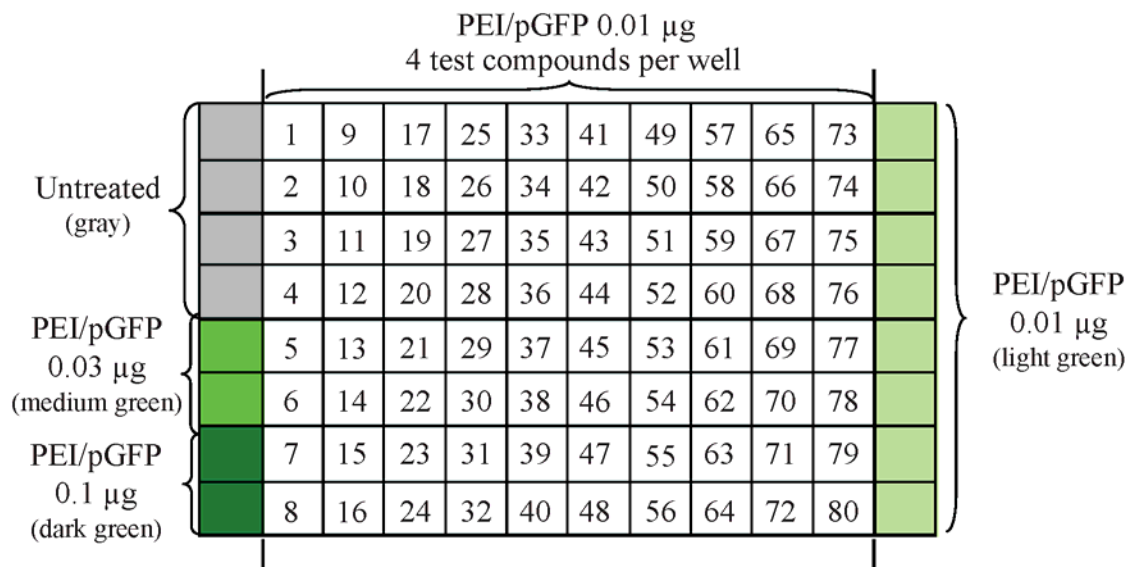
Figure 4-4 96-well layout for each plate in high throughput screen.
All wells contained 70-80% confluent B16F10 cells on 1st day of screen. Each plate had 80 test wells and 16 control wells. Each test well (unshaded) received a mixture of four test compounds (final concentration, 2.5 µM each) prior to transfection with 0.01 µg PEI/pGFP polyplexes. The control wells were either untreated (gray) or transfected with the indicated dose of PEI/pGFP polyplexes (green). The top four wells on the left side (untreated) are used to calculate background fluorescence for each plate. The eight wells on the right side are used to calculate baseline fluorescence for each plate.

4.4.2 Screen

An automated screening assay was designed that incorporated the preliminary results above and utilized an advanced robotic liquid handling station and labware transport arm along with a CO_2 incubator and two fluorescent 96-well plate readers (see methods). Figure 4-4 shows the layout of each 96-well plate used in the screen. In each well, ten thousand B16F10 cells were seeded and incubated in media with 10% serum for 24 hours prior to addition of the compounds or complexes, at which time the cells were 70-80% confluent. On each plate, 80 wells (test wells) were used for testing compounds and 16 wells are used for controls. Each test well received a mixture of four compounds from the library (final concentration of 2.5 µM each) 30 minutes prior to the addition of the complexes to the wells. The high throughput robotic system has a capacity of 40

76

plates. Thus 320 compounds can be tested on each plate and 12,800 compounds can be tested in each full-scale screen. Cells in all 80 test wells and 8 of the control wells were transfected with the baseline dose of 0.01 µg of DNA in 1.8/1 (+/-) PEI/pGFP complexes. Four addition wells were left untreated, two wells were transfected with 0.03 µg and two wells were transfected with the positive control of 0.1 µg. Following the 24 hour transfection, gene expression was quantified by measuring the fluorescence in each well using optimized fluorometer settings.

To investigate the utility of the high-throughput screen, we first performed small-scale and medium-scale screens of 1600 and 6080 compounds, each. Following each run of the screen, each well was assigned a score based on its fluorescence compared to internal controls on each plate according to the following equation:

$$Score = (test - background)/(baseline - background),$$

where test is the fluorescence in the test well, background is the mean fluorescence from four control wells of untreated cells and baseline is the mean fluorescence from eight control wells of baseline PEI/pGFP-transfected cells unexposed to compound. A histogram of the scores from each test well allows for the identification of positive outliers that have fluorescence greater than three standard deviations above the mean score, assuming a normal distribution. Figure 4-5 is a histogram of the results from the medium-scale screen with the mean and standard deviations indicated. The mean score is ~0.5 instead of 1.0 as expected because the compounds are in DMSO which could inhibit gene transfer and/or expression. In future studies, control wells should receive an equivalent amount of DMSO to overcome this issue. Of the 1,512 wells tested in this

screen (6,048 compounds), 33 had fluorescence greater than 3 standard deviations above

the mean and were thus designated as "positive hits". A similar hit rate was measured for

the small-scale screen.
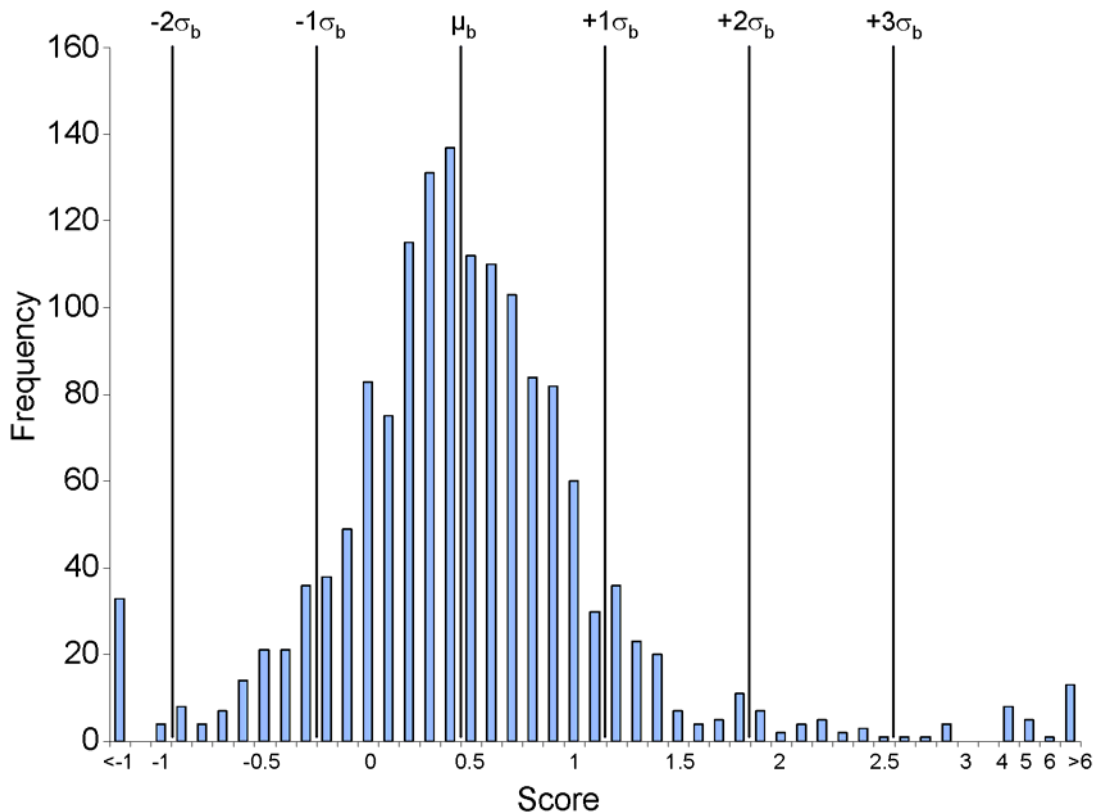
Figure 4-5 Histogram of scores from test wells of medium-scale run of screen.
Following a run of 19-plates in the high throughput screen, a score (as defined in the text) is
calculated for each test well (1520 total wells). Wells with fluorescence more than three standard
deviations above the mean are analyzed visually for false positives caused by compounds with
intrinsic fluorescence.

To determine if the increased fluorescence in each positive hit was due to an

increase in GFP expression and not intrinsic fluorescence of the test compounds, these 33

wells were observed by fluorescence microscopy. In each case, the increased

fluorescence was due to the compound itself and thus each of these wells represent false

positives. The fluorescent compounds generally fall into three categories (Fig. 4-6): (1)

"cell stainers" that adhere to cells (~50% of false positives), (2) "precipitators" that stain

the bottom of the well (~10%), and (3) "fibers" or "crystals" that form large macroscopic aggregates (~40%). The total rate of false positives is $33/6048 = {\sim}0.5\%$, which corresponds to 60-70 false positives for a full 40-plate screen and ~500 false positives in a complete screen of the 100,000 compound library. Since screening positive hits for fluorescent compounds can be done quickly with a conventional fluorescent microscope, this rate of false positives is sufficient for high-throughput screening.
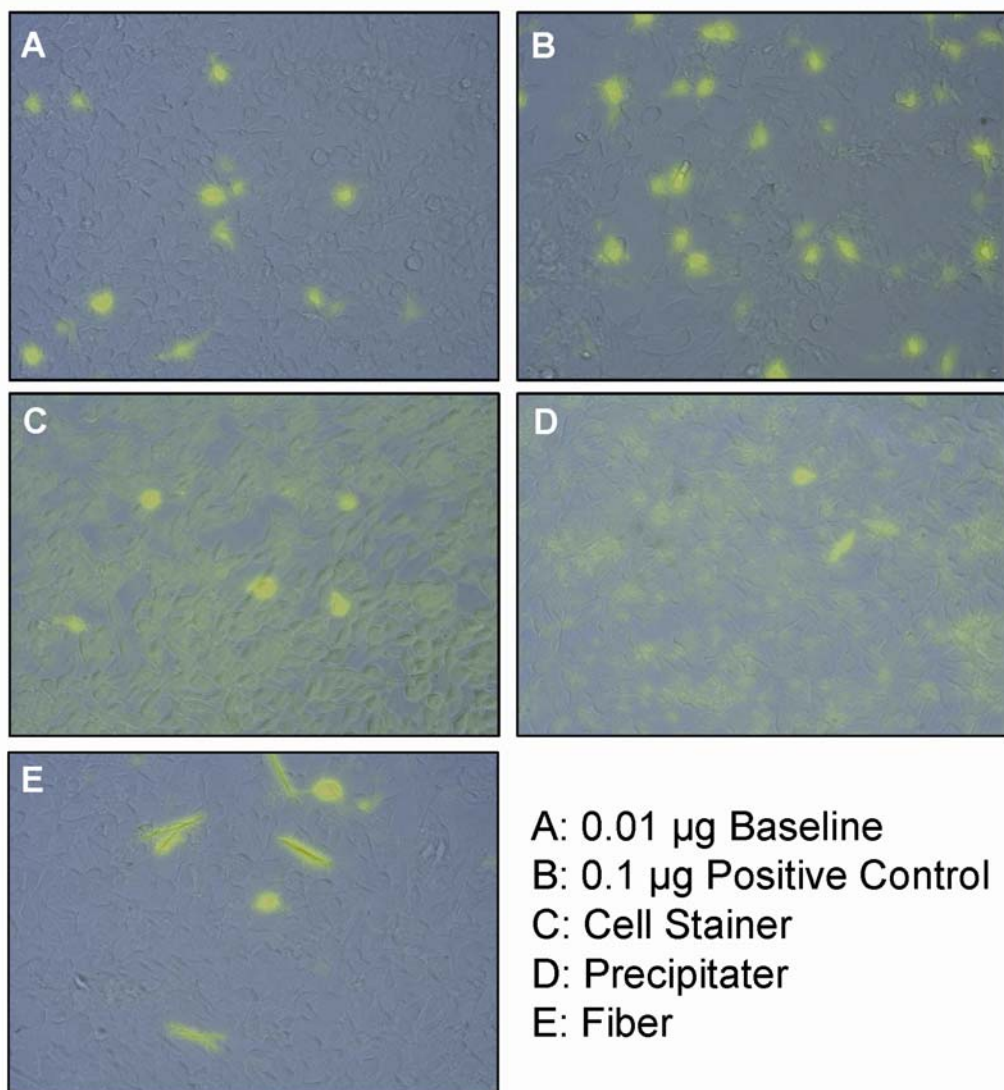
Figure 4-6 Fluorescent images of baseline and positive control transfections and common false positives.
B16F10 cells in a 96-well plate were transfected with the baseline dose of 0.01 µg DNA (A, C-E) or the positive control dose of 0.1 µg DNA (B). Panels C-E are examples of common false positives from test wells treated with library compounds prior to transfection.

4.5 DISCUSSION

We have designed a high-throughput assay to screen a large chemical library of low molecular weight compounds to identify molecules that enhance gene transfer mediated by non-viral vectors. In the original screen we have elected to screen B16F10 cells transfected with polyplexes consisting of PEI and a GFP plasmid. B16F10 is a cell type that grows in a rapid, reproducible manner, is adherent to the cell culture plate and can be readily transfected. PEI is a commercially available polymer that is widely used for *in vitro* transfections [143, 144] but has been shown to be toxic *in vivo* [145, 146] and thus would benefit greatly from improved transfection efficiency. GFP is a useful reporter gene for high throughput screening because no post-expression processing is required to measure the GFP signal [147, 148]. These properties enable a robust, high-throughput screen, though the techniques employed in this assay could be applied to any adherent cell line, transfection vector, and reporter gene.

A widely accepted and useful parameter for accessing the ability of a high throughput screening assay to identify active compounds is the Z-factor [149]. The Z-factor aims to quantify the overlap of baseline and positive control values, taking into account both the dynamic range and the data variation of the assay. Assuming that baseline transfection of 0.01 µg DNA without an enhancer compound results in a normal distribution of expression, a mean (μ_b) and standard deviation (σ_b) can be defined to describe this distribution. The same can be done for the positive control cells transfected with 0.1 µg DNA (μ_{c+}, σ_{c+}). The dynamic range is then defined as the difference in the means ($\mu_{c+} - \mu_b$) and the separation band is defined as the difference between values three

standard deviations below the positive control and values three standard deviations above the baseline ($[\mu_{c+} - 3\sigma_{c+}] - [\mu_b + 3\sigma_b]$). The Z-factor is simply the ratio of the separation band to the dynamic range and is therefore less than or equal to 1.

Zhang et al. [149] define an excellent assay as one with a Z-factor of 0.5 to 1 and an okay or "double" assay as one with a Z-factor of 0 to 0.5. If the Z-factor is 0 or lower, the signal distribution curves touch or overlap and the assay is not usable. The calculated Z-factor for the medium scale screen (test well distribution shown in Fig. 4-5) is 0.36. This value could be an underestimate because we do not have a known enhancer of PEI transfection and are thus using as the positive control a dose ten times higher than baseline (0.1 vs. 0.01 µg), which does not necessarily represent the maximum possible GFP expression.   For example, if the true positive control were just twice the expression from 0.1 µg, the Z-factor would be 0.55. We therefore conclude that the assay we describe is suitable for identifying small-molecule enhancers of transfection.

A common cause of false positives in our high throughput assay is the intrinsic fluorescence of a subset of the test compounds (Fig. 4-6). These false positives can be easily identified by observation with a fluorescent microscope, as described in results. Two alternative strategies should be noted that would eliminate the need for manual visual inspection. First, each compound could be tested in two wells, one in which the cells are also exposed to transfection complexes (as in the current assay) and a second in which the cells are not transfected. Intrinsic fluorescent false positive molecules would be detected as those that increase the fluorescence of both the transfected and

untransfected wells and could therefore be eliminated from consideration. The disadvantage to this approach is that twice as many wells are required which doubles the number of plates required for a certain number of molecules to be screened. Secondly, recent advances in high-content microscopy screening and image processing [150] have made it possible to include the visual false positive test into the automated screen with a microscope capable of reading 96-well plates and a processor capable of detecting cell stainers, precipitators, and fibers or crystals. The disadvantage to this approach is simply the added complexity. The addition to the screen of either of these alternatives depends on the perceived level of inconvenience of the manual microscopy test, which is proportional to the number of total molecules screened.

Transfection-enhancing molecules may act at three temporally distinct phases of the transfection process: Early, Middle or Late. The early phase consists of processes related to vector binding to the cell, vector internalization and endosomal escape of the pDNA. These early events occur within 60 minutes of the addition of the complex to the cells [6]. The middle phase is related to the transport of the DNA through cytoplasm and nuclear entry [151] which occur between 60 minutes and 6 hours after addition of transfection complexes to the cells. The late phase is identified with gene transcription from and nuclear retention of the plasmid DNA. We believe these late events occur between 6 and 36 hours after addition of the complex to the cells. The initial screen described here applies the library molecules and the transfection reagent sequentially, so it is applicable to identify compounds that can enhance the early steps in gene transfer. However, compounds that may enhance the middle and late steps in the process could interfere with

the early steps. If this is the case, the conditions optimized to identify early enhancers would fail to pick up late enhancers. To identify middle enhancers the library molecules could be added to the cells 60 minutes after the transfection complexes. In a similar fashion, late enhancers could be identified by adding the library molecules to the cells 10 hours after the addition of the transfection complex.

True positive hits determined by the high throughput assay should be further screened with the following steps. First, general transcription/translation enhancers can be eliminated by performing the same transient GFP transfection assay described here but on cells stably transfected with red fluorescent protein (RFP). Compounds that then increase the expression of both GFP and RFP can be considered general enhancers of gene expression without a specific effect on the transfection process. Second, these compounds should be re-screened at a range of doses with luciferase as the reporter gene. Luciferase has a higher dynamic range, allowing for better understanding of the compound's dose response, but requires the addition of a substrate to measure the transgene expression, limiting its usefulness in a high throughput screen. Third, a focused library of compounds structurally similar to potent transfection enhancers should be screened to establish structure-activity relationships (SAR) and to potentially identify compounds with even higher activity or potency. Finally, experiments should be performed to determine the specific transfection step that is affected by the enhancing compounds. These include investigating the effects of the enhancers on complex stability, cell uptake, endosomal release, cytoplasmic motility, and nuclear import. In addition to identifying enhancing compounds that could improve the efficiency of gene therapy,

these mechanistic experiments could help to identify which step in the transfection process is rate-limiting.

A full-scale run of the screen described in this study represents a high risk/high reward endeavor which is justified by the numerous examples of low molecular weight molecules that enhance gene transfer from non-viral vectors in cells or animals. The high-throughput screen we have described is a simple and robust assay that could lead to the discovery of simple drug-like molecules that will enhance the efficiency of non-viral delivery systems to the point of commercial reality.

4.6 ACKNOWLEDGEMENTS

CHAPTER 5: Conclusions

5.1 OVERVIEW

The focus of my PhD research has been on non-viral gene therapy at the cellular level. As a bioengineer, I have been drawn to drug delivery, and more specifically gene therapy, because I feel that fundamentally these fields represent biological problems with engineering solutions. Effectively, drug delivery scientists aim to engineer tools capable of delivering a drug from the point of entry into the body to the point of action. Furthermore, the design of these tools is aided by the use of gauges that allow the engineer to understand the existing biological system through which the drug will travel and how well that tool is working within the system. In the case of gene therapy, the design of the gene vectors ("tools") is aided by quantitative assays, microscopy, etc. ("gauges") that allow for a thorough understanding of the biological system.

This process is akin to the building of a bridge over a deep gorge. The aim of the bridgemaker is to build a tool (the bridge) that will deliver people from one side of the bridge to the other. In the process of designing this tool, the bridgemaker must use gauges to understand the system that he is working with: the geological details of the gorge (composition and stability of the gorge wall, wind conditions, etc.) and the nature of the traffic that will cross (trucks, cars, pedestrians, frequency, etc). With this information, the bridgemaker can select which materials and designs are the most suitable for the specific situation. The work presented in this dissertation presents my efforts over

the last six years to design new tools and gauges to improve the efficiency of gene vectors.

5.2 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Chapter 2 describes the design of a new tool to transport nucleic acid drugs through the cytoplasm: BART peptides. The motivation behind this tool is that DNA is a very large drug that has very limited diffusion in the crowded cytoplasm which impedes the movement of the DNA to the nucleus. We hypothesized that BART peptides, containing sequences from proteins that bind to molecular motors, can be used to hijack endogenous motors in the cell to actively carry BART-conjugated cargo. Our experiments showed that a BART peptide can bind to LC8, a light chain of dynein, and that BART-bound nanospheres can be transported along microtubules by dynein in a reconstituted system. Although the current BART system may not yet be a robust enough tool to function inside of cells, the results presented here are an encouraging first step towards a tool to increase the mobility of delivered DNA to the nucleus.

As our understanding of dynein structure and function improves, further strategies for linking cargo to dynein will become available. Recent studies have shown that LC8 may not be a dynein specific component and may instead function as a molecular "hub", connecting many multi-protein complexes in the cell [152], which would limit the utility of LC8 as a dynein adaptor. Therefore future designs of BART peptides should focus on other dynein cargo-binding subunits (eg. Tctex1 [86]). Alternatively, it may be possible that spectrin, which has been shown to link acidic liposomes to dynein/dynactin[153], could be used to mediate transport of liposome-encapsulated DNA. Although it is still

unknown whether or not increasing the cytoplasmic mobility of DNA will improve the efficiency of gene transfer, the answer to that question will add to our understanding of the significance of the cytoplasmic diffusion barrier.

The next step after diffusion of the DNA in the cytoplasm is entry into the target organelle: the nucleus. Chapter 3 describes the development and application of a "gauge" to quantify how much plasmid DNA is delivered to the nucleus of transfected cells: an internally controlled quantitative PCR assay of total DNA extracted from nuclei isolated from transfected cells. The development of this assay was motivated by the value of comparing nuclear delivery efficiencies between transfection vectors (in addition to the standard transgene expression) and by the lack of consensus on the amount of plasmid DNA delivered to a transfected nuclei. Our experiments showed that standard transfection doses of LFN lipoplexes or PEI polyplexes result in 75-50,000 plasmids per nucleus and that any increase above 3000 plasmids per 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. These results suggest that, for the cell types and vectors tested, strategies that consider the processes that may occur downstream from nuclear entry (transcription, vector unpacking, etc.) will have the greatest impact on gene transfer efficiency.

Although the nuclear delivery assay described here was established with two transfection vectors and two cell lines, the techniques used are easily transferrable to any

other vector and any other cell lines. Therefore this new "gauge" could be used to quantify the nuclear delivery of new vectors in development, providing an additional data point to the typical metrics of transgene expression and (sometimes) cell uptake. It would be interesting also to measure the nuclear delivery efficiencies of various vectors in non-dividing cells, which do not allow the delivered DNA to "sneak" into the nucleus during cell division. Similarly, the nuclear delivery assay should be very useful to prove that vector modifications rationally designed to improve the delivery of DNA to the nucleus are actually working as intended. For example, if it could be shown that a BART peptide can mediate active transport of DNA in the cytoplasm, one would want to know if this increased mobility translates to an increase in delivery to the nucleus on its own and/or with the addition of a nuclear localization signal (NLS) peptide.

Chapter 4 describes a high throughput cell-based screen for the discovery of small molecule enhancers of transfection. This is a different kind of "tool" than those described above: an enabling "tool" (screen) to aid in the discovery of working "tools" (enhancing molecules). Conditions were determined that allow for a simple, robust fluorescence screen capable of detecting transfection enhancing molecules, if present, in a library of drug-like small molecules. Medium-throughput runs of the assay established visual secondary screening steps that quickly eliminate false positives that result from intrinsic fluorescence of the test compounds.

The advantage of small molecules over typical components used in gene vectors (polymers, lipids, peptides, etc.) is that they are cheaper and easier to produce in a large

scale and are easier to deliver. Therefore the discovery of small molecule transfection enhancers could simplify non-viral vectors. The high-throughput screen should be used to fully screen our available library of small molecule enhancers and true positive hits should be further investigated according to the recommended steps in the discussion of Chapter 4. The goals of these follow-up steps are to develop molecules with even higher enhancing activity and to study the mechanism of action of the strong enhancers, which could aid in our understanding of rate-limiting steps in the transfection process.

5.3 LONG TERM OUTLOOK OF GENE THERAPY

Gene therapy still has the possibility of revolutionizing medicine. However, the long delay on the promise of nucleic acid therapeutics has led many skeptics to question whether gene therapy will ever be a reality. (My joke, **"**Gene therapy: five years away for the last twenty years.", always goes over well when I tell it at conferences.) This delay is not necessarily unexpected though considering that nucleic acid drugs present not only different mechanisms of action than most currently existing drugs but also different chemical characteristics: large size (~10,000x larger than small molecules), very hydrophilic, and very susceptible to enzymatic degradation. The "newness" of nucleic acid drugs can be compared to antibody drugs, which also took decades to develop but are now a multi-billion dollar industry.

Indeed, there continues to be exciting advances in the gene therapy field. The production, targeting, and safety of viral vectors is being improved [154]. Complete removal of CpG motifs from plasmid DNA is shown to drastically increase the duration of expression of non-viral vectors while also reducing immunogenicity [155]. Small

interfering RNAs (siRNAs) that can silence gene expression in primates are being developed [156] and have two significant delivery advantages over plasmid DNA: (1) they are smaller (closer in size to a protein) and (2) their site of action is the cytoplasm, eliminating the need for transport into the nucleus. Furthermore, hundreds of clinical trials are ongoing and the field is making a concerted effort to replace early hype with the rational stepwise progress needed to make gene therapy a clinical reality [157, 158]. Combining these advances with our increasing understanding of the barriers of gene transfer, I can't help but think that gene therapy's "five years away" may be closer than ever.

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ADDENDUM: OPPORTUNITY ANALYSIS FOR NEEDLE-FREE INJECTION IN THE DOMINICAN REPUBLIC

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This section is the final report from a field research study on the feasibility of implementing needle-free injection technology in the Dominican Republic that I was a part of from 2005 through 2008. The Bridging the Divide Program at UC Berkeley, which funded groups of graduate students to travel to other countries to study the application of technology developed in the United States to issues affecting the developing world, was the initial funding source and inspiration of this project. The research was an excellent opportunity for me to study drug delivery with a completely different perspective than the rest of my graduate work... working and speaking with end users of the technology to understand how devices should best be designed and implemented to meet their needs. This report was distributed to key stakeholders in injection safety worldwide and in public health in the Dominican Republic.

Opportunity Analysis for Needle-Free Injection in the Dominican Republic

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EXECUTIVE SUMMARY

Unsafe needle injections are associated with the spread of blood-borne infectious disease. More than 10 million people worldwide are infected with hepatitis B/C or HIV each year due to unsafe needle use resulting from accidental needle pricks, needle reuse, and improper sharps waste disposal (1). Needle free injection (NFI) delivers immunizations via a thin high-speed liquid stream, which penetrates the skin and carries the drug, eliminating the need for a needle. This technology reduces the negative consequences incurred from using needles including biohazard sharps waste, inadvertent needle sticks, and needle reuse.

In this report we present our June 2007 field study to assess the opportunity for using NFI for immunizations in the Dominican Republic (DR). Our three primary objectives were:

1. Determine if NFI is an appropriate technology for the DR

2. Gather feedback for design requirements for an NFI device to be used in the DR

3. Propose an implementation plan for NFI adoption in the DR

The study was conducted in Santo Domingo and Peravia in the DR with the support of the DR Expanded Programme on Immunization (Programa Ampliado de Inmunización, PAI). Sixty-one subjects were interviewed individually or in small groups, including health care workers (HCWs), local and provincial administrators, national policy makers, and international organization representatives.

Over 80% of the DR's 9.3 million people receive their healthcare through the public health system (Secretaría de Estado de Salud Pública y Asistencia Social – SESPAS). As a part of SESPAS's public health effort, PAI administers several million vaccinations a year through its 1250 vaccination clinics and vaccination campaigns. PAI independently and directly manages all immunization operations, including procurement, distribution, cold chain management, training, supervision, and injection of vaccines. Supplies are paid for by SESPAS through the Revolving Fund, a cooperative purchasing program managed by the Pan American Health Organization. Our interview subjects identified several challenges with regards to vaccinations and injection safety: increasing overall vaccination coverage, improving the waste management system for biohazard sharps disposal, eliminating needle recapping after use, and reducing accidental needle pricks to patients, immunization personnel, and disposal staff.

Objective 1: Determine if NFI is an appropriate technology for the DR

PAI's mission is to prevent the transmission of infectious disease by immunizing as many people as possible in an affordable and safe manner. To determine if NFI is an appropriate technology for the DR, we determined the following areas as key discussion points as they relate to NFI: injection safety, immunization coverage, affordability, sustainability, and device effectiveness. Subjects perceived that NFI would improve injection safety through elimination of sharps waste. They also thought that NFI would increase immunization coverage due to reduced fear of injection. Through an initial cost model, we show that costs are comparable between needle and syringe (NS) and NFI injections and that there are significant cost savings through reduced-dose delivery

(pending clinical studies) and inclusion of reduction of disease transmission in the model. From our discussions, we believe that it will be possible to communicate the benefits of NFI to HCWs throughout the DR to help make NFI a sustainable initiative. Furthermore, the DR PAI recently demonstrated the ability to successfully integrate a new technology into the immunization system. With previous data showing NFI immunization efficiency and a pending pilot study of NFI introduction in Brazil, it would be reasonable to predict that NFI will be effective for delivering all immunizations in DR.

Objective 2: Gather feedback for design requirements for an NFI device to be used in the DR

Subjects were very positive overall to both the concept of NFI and the specific devices we brought. People identified key benefits as: elimination of accidental needle pricks, safer waste management, and potential increased coverage. They were primarily concerned with device effectiveness, cost, cold chain maintenance, sterility, and the complexity of the injection process. These issues must be addressed with design modifications and further studies. From their feedback, we determined the main design requirements as: simple device preparation and injection, integrated trigger safety, disposable cartridge, maintenance-free device, and non-electric powering mechanism. Performance requirements are: proven clinical effectiveness, cost and time competitiveness with NS, compatibility with current infrastructure, and able to deliver intramuscular, subcutaneous, and intradermal vaccines.

Objective 3: Propose an implementation plan for NFI adoption in the DR

Any major decision involving PAI will require the consultation and approval of the following: PAI, SESPAS, and the Interagency Coordination Committee, a committee of representatives from the major international development agencies involved in the DR. We propose an implementation plan for NFI in which financing mechanisms, device procurement, additional training, patient education, and waste management are discussed.

During our trip, we also discovered a useful corollary in the 2001 introduction of the pentavalent vaccine alongside AD syringes into the immunization program. While the pentavalent vaccine is still used today, the AD syringes have been discontinued. Administrators introduced the vaccine after a burden of disease study showed that the prevalence of meningitis warranted its use. The Japanese government helped with the initial financing of the vaccine and the associated supplies, supporting 100% of it in the first year with a 20% drop-off each year until SESPAS paid for the entire cost. While the pentavalent vaccine proved to be very valuable to PAI, a majority of HCWs disliked the AD syringes, and its additional cost and perceived lack of need doomed their success.

NFI has the promise of solving many problems faced by the DR and other resource-challenged countries. Continuing forward, several items must be completed prior to a full-scale implementation of NFI in the DR: studies in the DR on the burden of disease due to unsafe injections and the potential increase in immunization coverage with NFI, a more detailed cost analysis for NFI use in the DR, additional clinical trials showing device effectiveness, and a successful pilot introduction of NFI in the DR showing device acceptance and usability.

LIST OF ACRONYMS

AD   Auto Disable [Syringe]
CDC   Centers for Disease Control and Prevention
DR   Dominican Republic
HCW   Health Care Worker
ICC   Interagency Coordination Committee
ID   Intradermal [Injection]
IM   Intramuscular [Injection]
JICA   Japanese International Cooperation Agency
MD   Medical Doctor
NFI   Needle-Free Injection
NS   Needle and Syringe
PAHO   Pan American Health Organization
PAI   Programa Ampliado de Inmunización (Expanded Programme on
            Immunization)
PATH   Program for Appropriate Technology in Health
SC   Subcutaneous [Injection]
SESPAS   Secretaría de Estado de Salud Pública y Asistencia Social (Ministry of
            Health and Social Assistance)
UC   University of California
UNICEF   United Nations Children's Fund
USAID   United States Agency for International Development
WHO   World Health Organization

INTRODUCTION

Motivation

Global immunization coverage has expanded greatly since the introduction of the Expanded Program on Immunization by the World Health Organization (WHO) in 1974. As the prevalence of vaccination programs in developing countries continues to rise, it becomes increasingly important to ensure that all injections are given in a safe manner to prevent the transmission of disease from patient to patient and from patient to provider. Several studies have shown that needles, which are currently used for almost all immunization programs and medical injections, can serve as a vector for infectious disease (2, 3). In developing countries, it is estimated that more than 10 million people are infected each year with hepatitis B/C or HIV due to unsafe needle injections (1) leading to 1.3 million deaths and US$535 million in direct medical expenditures (4). There have also been multiple epidemiological case studies directly linking the outbreak of diseases with unsafe needle use (2). Many new infections result from the passage of small amounts of diseased blood – one millionth of 1 ml (for comparison, a typical injection is half of 1 ml) from patient to patient when a needle and/or syringe is reused without proper sterilization. Other hazards associated with needles are accidental contaminated needle pricks to caregivers and inappropriately managed biohazard sharps waste.

As a result, alternative means of vaccine delivery, which do not use needles and are inherently safer, have been sought. An ideal alternative to needle-syringe (NS) vaccine delivery should confer immunity equally as efficiently, limit the transmission of blood

and blood-borne pathogens from diseased patients, decrease the amount of biohazard sharps waste, and cost equal to or less than existing technologies. Needle-free injection (NFI) is one technology that is currently being targeted to meet these criteria.

Study Objectives

The goals of this field study are stated below:

1. Determine if NFI is an appropriate technology for the Dominican Republic (DR)
2. Gather feedback for design requirements for an NFI device to be used in the DR
3. Propose an implementation plan for NFI adoption in the DR

Our team of four University of California (UC) Berkeley/San Francisco graduate students traveled to the DR to conduct a feasibility study for potentially implementing NFI as a replacement for NS for vaccine delivery in that country. The research team worked with representatives from the DR Ministry of Health, Secretaría de Estado de Salud Pública y Asistencia Social (SESPAS), and the DR Expanded Programme for Immunizations, Programa Ampliado de Inmunización (PAI), to interview health care workers (HCWs), local and provincial administrators, national policy makers, and international organization representatives. We obtained a better understanding of the current immunization infrastructure, observed typical practices at vaccination centers, and solicited device design recommendations. Based on feedback from our interviews and discussions, we also developed a potential implementation plan for introducing NFI into the DR immunization system, and we identified potential issues/barriers for a successful transition to NFI.

In the following chapters, we present our understanding of the DR's public health system, NFI device design feedback, an analysis of various implementation considerations if NFI were to be introduced in the DR, and finally a relevant case study of a recent technology introduction into the DR immunization system. We close by discussing different options for improving injection safety in the DR

Background

Needle-free injectors

Figure A-1. **(A,B)** Typical NFI devices currently used for insulin and hormone delivery. **(C)** Schematic representation of a needle-free injection delivering liquid into the skin (courtesy of Bioject).

NFI devices (Figure A-1) utilize a vaccine stream that is accelerated through a small orifice the width of a human hair at speeds capable of penetrating the skin to deliver fluid directly into tissue, thus eliminating the need for a needle. This method can be used to deliver drugs intramuscularly (IM), subcutaneously (SC), or intradermally (ID) and represents a number of advantages over conventional needle use. First, by eliminating needles from the injection process, NFI devices significantly reduce accidental needle-

pricks. Second, the repeated use of used non-sterile injection equipment is eliminated with NFI devices designed with single-use cartridges. Third, NFIs greatly reduce the amount of biohazard sharps waste and make disposal safer. Lastly, NFIs potentially could be used to consistently deliver vaccines ID. ID delivery of vaccines is capable of eliciting an immune response with as low as 20% of the vaccine that is currently used for IM and SC immunizations and could result in significant cost savings. The primary disadvantages of NFI devices are a higher price compared to NS, the potential additional resources needed for device maintenance, the limited volume that can be injected (<1 ml), and the risk of skin damage or laceration if the device is used improperly.

NFI development

NFI was first introduced in the 1940s for mass immunizations of military personnel. These devices used multi-dose syringes capable of immunizing many people in a short amount of time. In recent years, healthcare professionals and international organizations seeking to improve injection safety have increasingly endorsed NFIs with single-use, disposable cartridges. For example, the Bill and Melinda Gates Foundation has listed the development of needle-free delivery systems for vaccines as one of its 14 "Grand Challenges in Global Health" (5). Furthermore, the WHO reports that it "is seeking consensus from immunization partners regarding the design criteria and specifications for [needle-free] jet-injectors for immunization, and is communicating these to manufacturers..." (6).

<u>Current uses</u>

NFI devices (Biojector 2000, VitaJet, SeroJet, Injex, Pharmajet, and MediJector to name a few) are currently commercially available for personal delivery of insulin and human growth hormone. Also, some vaccinations centers in the United States use NFI devices for routine immunizations. However, these devices are currently too expensive and not practically designed for use in developing world environments.

Other NFI studies

This is the first study of this type in the DR, but other investigations in countries such as India, China, South Africa, Tanzania, and Brazil have also focused on NFI appropriateness and implementation. Both device manufacturers and non-profit organizations such as the Program for Appropriate Technology in Health (PATH) have investigated device design improvements for use in developing world environments (7). In addition to these studies, various clinical studies over the last two decades have shown that NFI immunizations confer immunity with similar or better efficiency when compared to NS injections (8-10). Other clinical studies have also provided feedback on the pain and usability of NFI compared to NS (9). Finally, the Centers for Disease Control and Prevention (CDC) is currently administering a study in the DR at the Hospital Robert Reid Cabral in Santo Domingo aimed at elucidating the effectiveness of NFI ID influenza vaccines using 40% of the standard dosage volume (11).

Alternative technologies to improve injection safety

As stated above, safe vaccine delivery is a global health challenge and a significant amount of international research is devoted to pursuing other technologies such as microneedle array drug delivery, aerosol technology, and single-use auto-disable (AD) syringes. These technologies are discussed in numerous academic articles, and an extensive bibliography is provided in the articles by Mitragotri (12) and Levine (13).

METHODS

Research Team

The core research team was comprised of four graduate students in the UC Berkeley/San Francisco Graduate Group in Bioengineering who designed and executed the research study: Michael Rosenbluth, Richard Cohen, Sapun Parekh, and Azucena Rodriguez. In addition, the UC team was assisted by three DR medical students: Luis Caminero, Carol Figueroa, and Georgina Polanco. The faculty advisors for the study were Dr. Glenny Guzmán, MD, Researcher in clinical research at the Departamento de Enfermedades Infecciosas at the Hospital Infantil Dr. Robert Reid Cabral in Santo Domingo, and Professor Dan Fletcher, Ph.D, from the Bioengineering Department at UC Berkeley. The research was funded by grants from the UC Berkeley Center of Integrated Nanomechanical Systems, the UC Berkeley Bridging the Divide program, the UC San Francisco Graduate Division, and the California Institute of Quantitative Biology.

Interview Subjects

During the field research in the DR, a total of 61 people were interviewed. We classified each interview subject into one of four groups by their occupation/expertise: health care workers (HCWs), local and provincial administrators, national policy makers, and international organization representatives (summarized below in Table A-1). HCWs included any person with experience administering vaccinations. Local and provincial administrators included PAI coordinators, SESPAS staff, and epidemiologists that immediately oversee healthcare practices at the neighborhood and municipality level. National policy makers included those working at the national SESPAS and PAI offices who are involved in healthcare policy and administration. International organization representatives included persons working at organizations that are in some way involved in the immunization program in the DR. The interviews occurred in and around Santo Domingo or Peravia province and were in most cases conducted at the subjects' place of employment.

Groups	Job Function	# of subjects
HCWs	Vaccination nurse	14
	Medical student	3
	General nurse	2
	Campaign manager	2
Local and provincial administrators	PAI coordinator	7
	Epidemiologist	3
	SESPAS staff	8
	Hospital director	4
National policy makers	PAI administrator	8
	Subsecretary of collective health	1
	SESPAS Technician	2
International organization representatives	Program coordinators	7
Total interviews		61

Table A-1. Breakdown of interview subjects by expertise and occupation.

Interview Protocol

Interviews were conducted in focus groups or individually depending on the availability and preference of the interview subjects. One or two investigators interviewed the subjects. All communication with the subjects was either in Spanish via a translator or English at the preference of each interview subject. The interview responses were recorded in English either as spoken directly from the interviewee or via a translator. Informed consent was obtained from each participant prior to any data recording. The basic interview procedure following informed consent is outlined in Figure A-2 below:
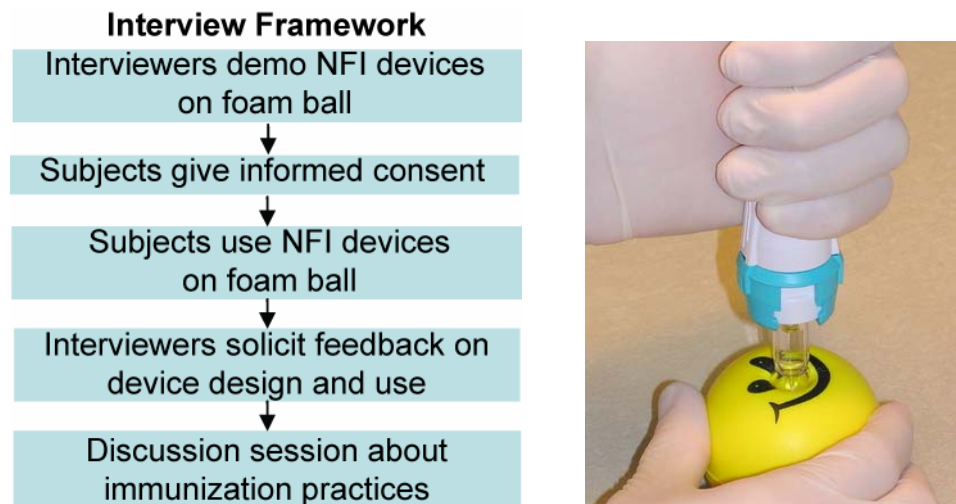
Figure A-2. (Left) Basic format used during interviews. (Right) NFI injection into foam ball.

Interview guides were used by the investigators to lead an open discussion.

Ethics Board Approval

We received approval from the Center for the Protection of Human Subjects at UC Berkeley and the Consejo Nacional de Bioética en Salud – the National Commission of Bioethics in Health in the DR.

Scope of Study

Any data presented in this report that is not specifically cited should be assumed to be a summary of the information provided to us by the interview subjects, as well as our own observations during the field study, and is presented to the best of our knowledge. Next, given that all of our interviews at immunization clinics in the DR occurred either in the provinces of Santo Domingo or Peravia, the responses from our HCW subjects at these clinics can only be extrapolated to the entire country to the extent that these clinics represent the DR's health system.

CURRENT IMMUNIZATION SYSTEM

In the following section, we describe how vaccines are administered through the public health system in the DR. We begin by providing a brief country profile and proceed to explain the top-down organization of the immunization system starting with SESPAS and PAI and concluding with individual vaccinations by HCWs at public vaccination facilities. This section of the report is not critical for understanding/interpreting the three study objectives and can be bypassed without loss of clarity.

Vaccine	Dose (mL)	Type of needle	Type of Injection	Patient Age When Given	Infant Coverage
Pentavalent (Hib, DTP3, Hep B)	0.5	23 G x 1" length	Intra-muscular	2, 4, 6 months	Not reported
MMR (SRP)	0.5	25 G x 5/8" length	Sub-cutaneous	12 months	61%
BCG	0.1	26 G x 3/8" length	Intra-dermal	Birth	97%
Hep B	0.5	23 G x 1" length	Intra-muscular	Birth	70%
DTP3 booster	0.5	23 G x 1" length	Intra-muscular	18 months, 4 years	81%
Polio	2 drops		Oral	2, 4, 6, 18 months and 4 years	70%

Table A-2. DR infant vaccine schedule (adapted from official PAI vaccine card). Coverage percentages are from 2004 data from PAHO (15).

Country Demographics

The total population in the DR is approximately 9.3 million with a growth rate of 1.5% every year (14). In 2006, there were 227,000 births, and approximately 7 million vaccine doses were procured for immunizations to be administered at the 1,250 public vaccination facilities. Table A-2 summarizes information about the specific vaccines recommended for newborn children and available free of charge at public vaccinations

centers in the DR. Also included in Table A-2 are the type of injection, dosage volume, and the recommended patient age at the time of administration for each vaccine.

Ministry of Health and Social Assistance (SESPAS)

Healthcare in the DR is administered by public and private clinics. In the present study, we focus primarily on understanding how the public healthcare system is organized because greater than 80% of the population in the country is treated at public clinics and hospitals. The governing body that oversees the entire public health system is SESPAS. This institution oversees all public health care in the country and coordinates funding on behalf of different aspects of the system. SESPAS is organized into 5 main directorates (Figure A-3), and PAI falls under the direction of the Secretary of Collective Health.
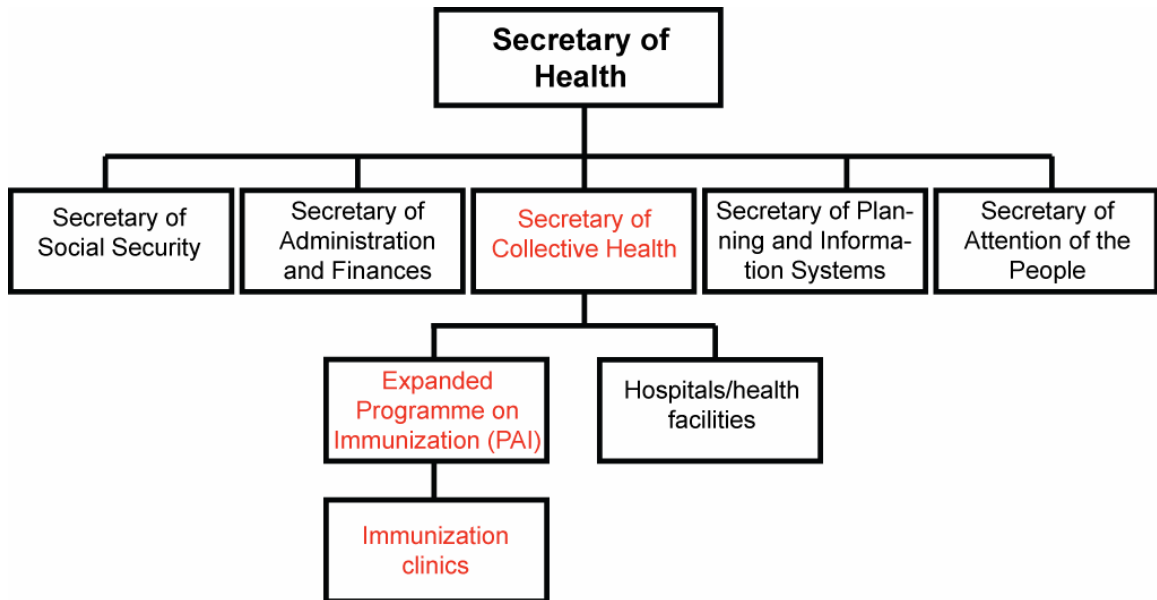
Figure A-3. SESPAS organization and position of PAI within the public health system.

Expanded Programme on Immunization (PAI)

As shown in Figure , PAI exists as part of SESPAS. It directly manages and regulates all public immunization clinics, whether they operate as part of hospital facilities or as

independent vaccination posts. As an organization, it coordinates administration, vaccine purchase, distribution, cold chain management, HCW training, and HCW supervision. PAI is a national level institution based in Santo Domingo and is divided into eight regions and 39 sub-levels including 8 areas in Santo Domingo and 31 provinces (Fig. A-4, Fig. A-5). 1250 public vaccination facilities serve the entire country. All healthcare operations are immediately regulated via the 4-5 provincial offices in each region. Population density determines the boundaries of the PAI provinces.
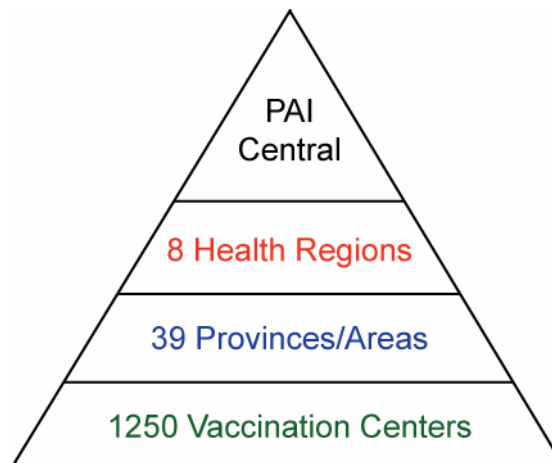
Figure A-4. Structural organization of PAI
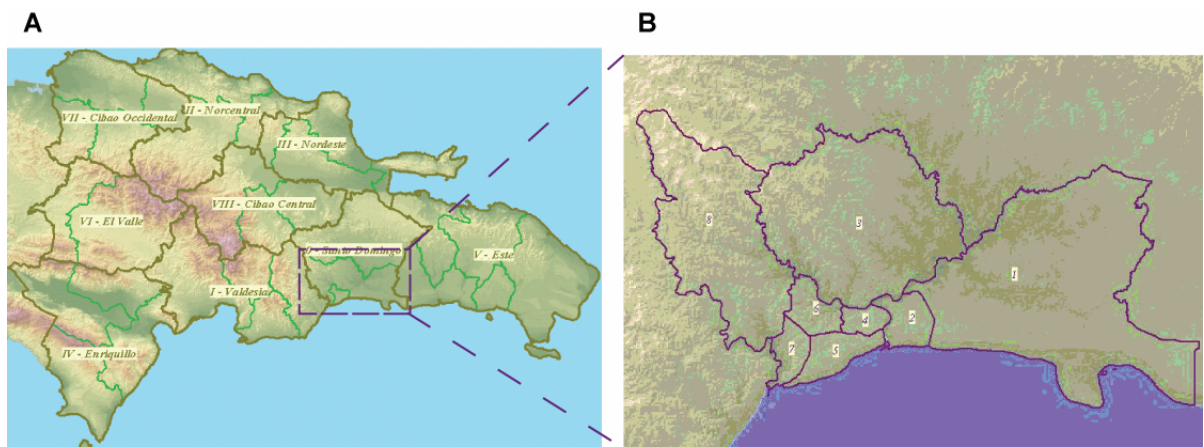
Figure A-5. (A) Map of DR showing 8 PAI regions (brown lines) and provinces (green lines). (B) Map showing 8 PAI Areas in Santo Domingo, which is in Region 0.

While 80% of healthcare treatment is received at public facilities, 95% of all vaccinations are administered by PAI/SESPAS facilities. Typically vaccination nurses provide immunization services. In larger hospitals, multiple nurses often work under a head vaccine nurse who is responsible for supervision and training of other nurses in the vaccine department.

Training and Supervision

Generally, the training of vaccination personnel for routine immunizations is administered in a top-down fashion where provincial/area PAI coordinators are trained at the PAI central office in Santo Domingo after which provincial PAI officers train the head vaccination nurses (or standard [auxiliary] vaccine nurses if there is no head nurse at a facility). These nurses then train (other) auxiliary vaccine nurses at their respective facilities. PAI national administrators train provincial personnel twice a year, but immunization nurses are given refresher training monthly by the provincial PAI coordinators.

PAI provincial coordinators supervise immunization practices at all immunization facilities in their provinces one to five times per month, depending on the province. Short refresher courses and instruction are often coupled with these supervisory visits. Supervisory information such as HCW compliance with PAI safety procedures, HCW injection practices, and resource consumption from each province within a region is compiled monthly, and the eight PAI regional supervisors meet with the national PAI director in Santo Domingo every three to four weeks to discuss this information.

Vaccination Campaigns

In addition to routine vaccinations in clinics, the national PAI and SESPAS offices organize campaigns to quickly "catch-up" the population in order to increase vaccine coverage of the recommended vaccines on the schedule (Table A-2). Each year in April, the DR participates in "Vaccination Week in the Americas" – an international vaccine campaign aimed at vaccinating anyone deficient for scheduled immunizations. In 2006, an additional campaign was implemented to protect against an outbreak of Rubella.

Numerous two-person teams were deployed in each area during a recent campaign. These teams consisted of one person that was in charge of giving immunizations and another that carried biohazard disposal boxes, immunization records, and other supplies. Immunization personnel for campaigns consist of vaccine nurses as well as many community volunteers and medical students.

Training for campaigns takes between one day and one week, depending on the medical knowledge of the personnel and technical difficulty of the medical process (i.e. oral versus injection vaccine delivery). Volunteer training is administered by immunization nurses and occurs in the municipalities and neighborhoods within each province and not necessarily at a PAI office.

Vaccine Pathway

From our observations and conversations with immunization nurses, PAI coordinators, and policymakers, we have summarized the route for vaccines from purchase to injection waste disposal.

Vaccine Financing

The PAI budget is set by submitting a five year plan to SESPAS, whose budget is set by the President and national congress. Each year the PAI leadership, along with a group of donor organizations (the Interagency Coordination Committee – discussed below), evaluates the country's immunization needs, and determines the final dollar amount to request from SESPAS. The PAI budget for 2006 was $386 million RD (~ $12.9 million USD) and an additional $800,000 RD (~$26,000 USD) was donated for the rubella campaign. All PAI recommended vaccines administered at public facilities are fully subsidized by SESPAS and free for patients.

Purchasing

The DR PAI, like in most other Latin American countries, purchases all vaccine supplies through the Pan American Health Organization (PAHO) Revolving Fund. These countries combine their immunization budgets into the PAHO-run fund, increasing their buying power. Furthermore, because PAHO administers the Revolving Fund, needle, syringe, and vaccine manufacturers must comply with a PAHO protocol/standard to be considered for purchase by the fund. This ensures that each country receives high quality vaccination supplies. The quantity purchased is based on projections of the population, executed by the census bureau, Consejo Nacional de Población y Familia.

Storage

Supplies are stored in the PAI central office in Santo Domingo and routed to different provinces in the country as necessary. The central PAI office houses four 4°C cold rooms for vaccine storage, two open-up freezers for frozen block storage (used for vaccine transport from central office to provincial offices), and a storage room for syringes, needles, thermoses, coolers, alcohol swabs, and safety boxes.

Distribution

PAI Coordinators from each provincial office come to the PAI central office at least once per month to retrieve all immunization supplies. These supplies are stored at provincial PAI offices and get distributed to hospitals and clinics in the province. Due to lack of sufficient refrigeration, smaller facilities must procure supplies often (i.e. as needed) while larger hospitals operate on a well-defined schedule. Immunization supplies provided by PAI are completely independent from supplies for medical/therapeutic injections.

Cold chain and handling

At each level of the supply chain, the cold chain is very carefully maintained to minimize vaccine vulnerability. Immediately after obtaining vaccines and other injection materials for immunizations from the PAI central office, provincial offices store vaccines in refrigeration units. Larger hospitals follow the same protocol upon receipt of vaccine vials from the provincial PAI offices. The refrigerators used for storage at both the provincial offices and hospitals operate on gas or electric power to allow for continued

cooling during electrical power outages. The temperature of these storage units is checked twice a day to ensure that an appropriate temperature (2-8°C) is maintained.

The typical practice at larger hospitals is to take a small, usable quantity of vaccine vials to the immunization clinic. Once removed from the refrigerators, the vials are placed in plastic thermoses containing freezer blocks to maintain a cold temperature. At some of the smaller facilities that do not have refrigerators, vaccines are always kept in thermoses and coolers because they have no capability for long term storage as mentioned above. Immunization campaign workers use the same practice of storing vaccines in coolers/thermoses. They expressed concern that vaccines will not remain cold in thermoses because of the tropical climate in the country.

Vaccine delivery

Most vaccines are given in the first year after birth. Table A-2 shown earlier lists the recommended vaccines in the schedule. Nurses in the immunization ward primarily administer vaccines to children. At larger facilities, one immunization nurse will often go to the maternity ward to vaccinate newborns while the others work in the vaccination clinic. Immunization nurses also vaccinate older children that have missed scheduled vaccines, and they will often give multiple immunizations in the same appointment ("auto-complete" the vaccine schedule).

The basic procedure for administering vaccines is to:

1) Wipe the target area (most often thigh/quadriceps) with an alcohol swab

2) Withdraw vaccine from the vial

3) Replace the vial in the cold thermos before giving the injection (for multi-dose vials only)

4) Inject patient with vaccine

5) Dispose of injection waste in biohazard containers

During campaigns, vaccine teams travel from house-to-house in certain areas and set up central vaccination posts in others. As stated above, campaigns are the primary method of "catching up" the population with the appropriate vaccines. In the future however, PAI hopes to eliminate vaccine campaigns by increasing vaccine coverage in routine immunization settings so that all newborn children are up-to-date.

Waste management

After an injection, the official protocol for waste disposal is to place the needle and syringe (NS) – uncapped – in yellow disposal boxes. These boxes, recommended by the WHO for sharps biohazard disposal, are generally located in the immunization ward. We occasionally observed nurses recapping needles after injections before disposing of the NS. This increases the risk of accidental needle pricks and violates the disposal protocol as stated by PAI and the WHO (16).

In the event that a facility ran out of disposal boxes, it is instructed to use old milk cartons or soda bottles as disposal containers (as specified by PAI and the WHO guidelines). Nurses often commented that boxes had overflowing contents before they were replaced. The janitorial staff is in charge of collecting and incinerating all sharps biohazard waste for the entire facility. Larger hospitals, such as Hospital Maternidad in

Los Mina, have their own incinerators and operated them daily for two hours while smaller clinics brought their biohazard waste to larger facilities for incineration. Exhaust fumes are vented to the ambient air, and the ashes/burned material are transported to the city dump location for disposal with all other garbage.

International Organization Support

Currently, SESPAS provides funds to purchase all consumable supplies for scheduled vaccines through the Revolving Fund. International organizations such as the Japanese International Cooperation Agency (JICA), United States Agency for International Development (USAID), PAHO, Project Hope, and the United Nations Children's Fund (UNICEF) support PAI by providing administrative support and capital equipment such as refrigerators, computers, and vehicles. We spoke with representatives from JICA, USAID, PAHO, Project HOPE, UNICEF to understand their specific involvement in the immunization system. See Table A-3 for a summary of the international organizations' primary involvement with DR PAI.

International Organization	Primary involvement with DR PAI
JICA	• Funding for pentavalent introduction • Provide support for capital equipment such as vaccine refrigerators and computers • Improve nurse training and education in Northeastern DR
USAID	• Coverage surveys in underserved regions • Training for cold chain maintenance and biohazard disposal in Region 5 • Sponsor DR for PEPFAR funding
PAHO	• Administer Revolving Fund for all vaccine supply purchases • Primary consultant to PAI for new vaccine initiatives and technology • Organize meetings with other organizations to discuss progress and challenges with PAI
Project Hope	• Direct support in procuring vaccines and consumables
UNICEF	• Support and funding for training, capital equipment, and education in remote locations • New technology consulting

Table A-3. International aid and donor organization involvement with PAI.

Interagency Coordination Committee

The Interagency Coordination Committee, consisting of representatives from JICA, USAID, PAHO, Project Hope, and UNICEF, among others, meets with the PAI and representatives of SESPAS to discuss the immunization coverage, budget, and future initiatives. PAHO coordinates ICC meetings every three months, where the different organizations present goals and strategies for helping PAI and together decide the best distribution of the funds/support. For example, before introduction of the pentavalent vaccine, the ICC met to discuss different support roles for organizations to assist JICA. In addition, the ICC is currently working with PAI to draft a vaccine law that will guarantee the PAI budget regardless of the desires and needs of other government programs. The vaccine law legislation will ensure that the budget for vaccines and supplies will be isolated from any tampering and will allow PAI to reliably purchase immunization materials each year.

OBJECTIVE 1: DETERMINE IF NEEDLE-FREE INJECTION IS AN APPROPRIATE TECHNOLOGY FOR THE DOMINICAN REPUBLIC

From our discussions with PAI, SESPAS, and donor organizations, the following are factors that need to be considered when deciding whether or not NFI is an appropriate and potential solution to the challenges and needs currently faced by PAI.

Injection Safety

NFI has the potential to improve injection safety in the DR in the areas of biohazard sharps waste management, reducing needle recapping, and minimizing inadvertent needle pricks. According to a recent study in 2005 and 2006 by Moro *et al.*, 10% of HCWs in immunization clinics reported a needle-stick injury (17). They found 12% of HCWs at immunization clinics recapped needles, and 15% of clinics had sharps waste discarded in regular waste or non-puncture proof containers (17).

While this study is indicative of problems with needle safety, our policy maker subjects often mentioned a desire for a formal study to confirm that the issue that NFI is intended to address – disease transmission resulting from unsafe injections – is a significant problem in the DR. This type of study would evaluate how many people in the DR are infected with blood-borne diseases as a result of unsafe injections with NS each year and how much money is spent caring for those people. Similar studies investigating the

global burden of disease due to unsafe injections have been reported (1, 3). However, a national study using similar techniques to those studies should be performed and should consider local estimates for disease prevalence and the probabilities of accidental needle-pricks to HCWs, patients, and disposal staff to confirm that a sufficient need exists for an NFI implementation.

Immunization coverage

After speaking with several high-ranking PAI officials, a consistent theme was that increasing immunization coverage in the entire country, especially in remote regions, was a goal of PAI. Some officials commented that using NFI instead of NS may increase vaccination coverage because people (particularly children) will not associate NFI with pain and fear as they do with needles and so are more likely to complete the vaccination schedule. The reduction of fear by eliminating needles was also seen as a very positive impact by HCWs because children often squirm and twitch when they see a needle, which creates a dangerous environment for the HCW, mother, and child. Using NFI would avoid this problem entirely as there are no preconceived notions about NFI injections though a similar fear of NFI devices could evolve over time if NFI technology becomes the standard for vaccination delivery.

Affordability

Cost was consistently raised as a key decision making factor by our interview subjects. The cost of NFI must not be significantly higher than NS (per immunization) otherwise NFI would not be a potential solution, regardless of its positive impact. Officials stated

that they would include the following parameters in a cost model: cost of devices and cartridges (supplies), training, and waste management; and possible savings from the reduced dose required for intradermal delivery with NFI devices. Officials also indicated that a reduction in disease burden should be included in the model.

	Current	NFI	NFI - Reduced Dose
Consumables	253,940	636,570	636,570
Vaccines	3,951,210	3,713,400	2,353,823
Waste disposal	300,960	153,360	153,360
Burden of disease	62,364	0	0
Total	**4,568,474**	**4,503,330**	**3,143,753**

Table A-4. Current estimates of the cost of vaccines, consumables, waste disposal, and the burden of disease for NFI as compared to the current system. Costs are per year for the entire immunization program. These estimates are for large-scale production, which would require implementation of NFI in several countries.

We have assembled a basic cost model in (summarized in Table A-4) to address the issue of NFI affordability, though a more in-depth analysis would need to be done with exact device costs to be more accurate. This model considers costs of an already implemented NFI program and does not incorporate start-up costs such as additional training and public awareness campaigns nor potential net savings due to increased immunization coverage. We estimate that the cost of consumables (i.e. syringes/cartridges) would more than double, but that total spending on vaccines and consumables would increase by only 3% due to less vaccine wastage and the relative low cost of consumables as compared to vaccines. Including cost of waste disposal, NFI and the current system are equivalent in cost (Table A-4) and if the monetary impact of burden of disease is included, NFI is ~ 1.5% cheaper than the current system. If reduced-dose intradermal delivery were to be implemented, a cost savings of 30% would be realized (31% including burden of disease).

Device effectiveness

Policy makers, administrators, and donors wanted to see clinical data that show that NFI produces equal or better immunogenicity than NS with each of the vaccines that PAI administers. If ID delivery with NFI is to be considered, then these studies must include results on ID delivery for each vaccine. Furthermore, all of these studies would need to be validated by PAHO and UNICEF. Similar efficiency studies were conducted prior to the introduction of the pentavalent vaccine to evaluate the effectiveness of Hib vaccinations on incidence of bacterial meningitis.

Multiple academic studies with the Biojector 2000 NFI system have demonstrated that influenza and hepatitis A and B vaccines delivered by NFI are equally or more immunogenic as those delivered by NS (8, 9). While the specifics of such studies are beyond the scope of this work, additional immunogenicity studies with other vaccines on the PAI vaccine schedule are a prerequisite to a large scale implementation of NFI in the DR. Additionally, clinical studies in developing countries will provide useful feedback on NFI device durability. Previous and future clinical data showing NFI immunization efficiency together with results of an upcoming pilot study/clinical trial of NFI introduction in Brazil by PATH will comprise a body of knowledge from which it should be reasonable to predict the effectiveness of NFI in the DR.

Sustainability

Many subjects noted that implementing a large-scale change in the health care system is difficult and a plan for sustainable funding, long term PAI commitment, and patient and

HCW education is needed to ensure that the integration of a new technology would be lasting solution. It was suggested that if HCWs are well trained and educated to the benefits of a new technology, they will be supportive, which would circumvent a major barrier towards making NFI sustainable in the DR.

From our discussions, we felt that most people understood the benefits of NFI. Thus we believe it will be possible to communicate the benefits to HCWs throughout the DR. Additionally, the recent introduction of the pentavalent vaccine, where the initial funding was subsidized by JICA and eventually fully assumed by PAI, has proven to be a great success (see case study on page 169). This recent success of a new vaccine technology introduction coupled with our finding that many HCWs quickly realized the utility of NFI leads us to believe that NFI will be a sustainable initiative for the DR.

NFI is appropriate for the DR

After evaluating the feedback we received from interviews, performing a cost analysis, and reviewing relevant literature, we believe that NFI is an appropriate technology upgrade for the DR immunization program and will have a positive impact on the factors presented above. Several actions should be taken before a decision is made on implementing NFI, which we summarize in Table A-5. We compare NFI against alternatives on page 177 to determine the best options for improving injection safety.

Issue	Action
Need	Formal study assessing increased immunization coverage with NFI and burden of disease caused by unsafe injections
Effectiveness	Clinical trials with NFI in the DR or similar settings
Affordability	Detailed cost model of NFI
Sustainability	Pilot introduction of NFI in the DR to assess use/acceptance

Table A-5. Further actions that should be taken before the DR would decide whether to approve NFI for immunizations.

OBJECTIVE 2: GATHER FEEDBACK FOR DESIGN REQUIREMENTS FOR A NEEDLE-FREE INJECTION DEVICE TO BE USED IN THE DOMINICAN REPUBLIC

In this section, we review the design feedback gathered from interview subjects. In the first part, we summarize the feedback on the general concept of NFI. In the next part, we detail the specific feedback subjects had on the prototype devices brought. Lastly, we create design requirements for a NFI device based on this feedback.

Feedback on the concept of needle free injection

General benefits and concerns of NFI

Most interview subjects were enthusiastic about the technology. They were excited about using new technology, particularly if it was a clear improvement. Feedback was varied across groups as well as within groups, but several common themes emerged. The key benefits mentioned were:

- Elimination of accidental needle pricks: vaccine delivery with NFI would be safer and less stressful for HCWs and patients.

- Safer waste management: No sharps in biohazard waste would be safer for HCWs and the general population which would be less likely to encounter biohazard sharps in the community.

- Increased coverage: NFI was perceived to be more appealing to patients and would encourage people to come in to clinics to get vaccinated and less likely to avoid HCWs during outreach campaigns due to fear of needles.

There were also several requirements/concerns that were mentioned by many people interviewed. These were:

- Effectiveness: The key question people asked was "Is this as effective as needles?". Solid clinical data needs to be provided on each vaccine type delivered with NFI to compare effectiveness versus NS. If NFI is to be used with reduced dosage, these studies need to show equal effectiveness to full-dose NS.

- Cost: This was mentioned as a key concern, even with people not involved in the actual purchase of vaccine supplies. Our economic model addresses this concern, but more detailed analysis should be done in the future.

- Maintenance of the cold chain: NFI devices were perceived as taking more time to inject than NS, which may disrupt the cold chain.

- Sterility: Several steps of the injection process with NFI were seen as difficult to avoid contamination.

- Simplicity of process: Keeping the injection process simple and reducing the number of parts were stated as important to gaining acceptance of NFI.

Each subject group tended to mention some points more than others. Table A-6 lists the key advantages, concerns, and priorities for the various groups.

Subject	Benefits	Concerns
Clinician	Safer for HCW Higher coverage	Awkward to hold Maintaining cold chain Process too complex
Regional Administrators	Safer for HCW Higher coverage No sharps waste	Maintaining cold chain Cost Contamination
Policy makers	Safer for HCW No sharps waste Potential volume reduction	Cost Effectiveness Effectiveness with 20% ID dose Consistency of device over time

Table A-6. Key benefits and concerns of NFI by subject type.

Specific feedback on the concept of NFI

Responses were then categorized based on the impact of NFI to the following specific immunization issues.

Aversion to needles

Much of the enthusiasm expressed about NFI by the interview subjects was related to reduction in the patients' fears of injection. Many people (children and adults) dislike NS injections and will not come to a clinic for needed vaccinations or will avoid HCWs coming to their neighborhood during campaigns. Many interview subjects indicated that the fear of needles is one factor that limits vaccination coverage in the DR. They felt that if needles were removed from the vaccination process, more people would come to be vaccinated and that this could increase vaccination coverage.

Disease transmission

Most interview subjects recognized that NFI would alleviate the risk of disease transmission from unsafe injections related to accidental needle pricks and sharps waste management. According to the subjects, needle reuse does not occur in the DR, and

would not be affected by NFI. Subjects recognized that like disposable needles and syringes, the disposable NFI cartridges were only to be used once.

Needle pricks

Many nurses said they had been pricked with a used needle, that they knew somebody who had, or that they knew that needle pricks occurred in the DR. However, because there is not a defined procedure for reporting needle pricks or obtaining treatment after one occurs, nobody knew exactly how much of a problem this was. Some of the nurses that were interviewed did express nervousness about getting pricked with a needle that had been used in high-risk patients, such as children with blood-borne diseases or undocumented persons from other countries. Also, there were some concerns that waste disposal personnel could be getting pricked during disposal of needles but data on that was also not available. The subjects we interviewed recognized that NFI would eliminate the risk of needle pricks during injection.

Waste management

Most subjects felt that NFI would greatly improve the medical waste management system in the DR and many felt this was the main advantage of NFI. The cost of waste management and the amount of disease transmitted from improperly disposed of needles could be greatly reduced if sharps disposal was eliminated.

Cost

The cost of NFI was a major concern for many interview subjects and typically one of the first questions subjects asked. Cost reduction through use of ID injections with reduced vaccine load was appealing to policy makers, though coupled with skepticism of equal effectiveness to traditional vaccine delivery. Subjects indicated openness to consider indirect costs that could make the price of NFI cost-competitive. These include (among others) the reduction of disease, easier waste management, and simplified training.

Contamination

Many interview subjects were concerned that an NFI device might require too much manipulation that could lead to contamination of the syringe or vaccine vial. HCWs do not use gloves in immunization clinics in the DR, increasing the contamination risk.

Cold Chain

Subjects were concerned that the vaccine vial would be exposed to room temperature for too long of a time while the syringe is loaded and that this would disrupt the cold chain. However, many agreed that these concerns could be addressed with proper training and device design dedicated for immunization practices.

Security of devices

There was only minimal concern that the devices would be stolen from vaccination clinics. Although it was recognized that theft is a possibility, most did not think this would be a large problem because the vaccinators typically get respect for what they do

and because everybody in the area knows each other. Generally, each vaccination clinic has a safe storage area where the vaccination refrigerators and excess supplies are kept.

Feedback on Needle-Free Injection Devices

In this section, we summarize the feedback on NFI devices from our field visits. We discuss the devices tested, document the feedback from participants that were pertinent to both devices, and present device-specific feedback.

Devices tested

Devices from two companies were used to demonstrate the technology to our subjects during interviews. These devices were not designed for use with immunizations nor in developing countries, and were presented to generate ideas rather than for device evaluations. Of note is that the subjects were only given the opportunity to try the devices 1-3 times, and the feedback will be affected from this bias.

Bioject provided the team with a variation of the Vitajet (Figure A-6 and Table A-7). A similar device is currently marketed in the United States for delivery of human growth hormone. The spring-powered device has three main parts—the injector, a single-use disposable cartridge, and the adapter to interface the cartridge with a vaccine vial. The spring is charged by manually twisting the knob. The device is discharged by pressing the button atop the knob.

Figure A-6. The Bioject device along with the cartridge, vial adapter, and a sample vial.

Bioject Device	
Dose	0.05-0.5 mL
Weight	160 grams
Power	Spring
Charge method	Winding spring, on device, manual
Lifetime	Several thousand injections
Device cost	To be determined
Part cost	Cartridge: To be determined Vial adapter: included

Table A-7. Details on the Bioject device tested in the DR.

Injex provided the team with two devices—the Injex30 and Injex50 (Figure A-7 and Table A-8). These devices are currently marketed in Europe for insulin use. The main difference between the two devices is the maximum delivery dose – the Injex30 delivers up to 0.3 mL while the Injex50 delivers up to 0.5 mL. Feedback on the Injex devices was largely similar and therefore pooled. This device, consisting of the injector, single-use disposable cartridge, vial adapter, and charging box, is also spring powered. To prepare for an injection, the injector is first loaded into the charging box. The lid of the charging box is then shut on the device, charging the spring. The device is discharged by pushing down the safety collar and pressing down the button, located on the side.
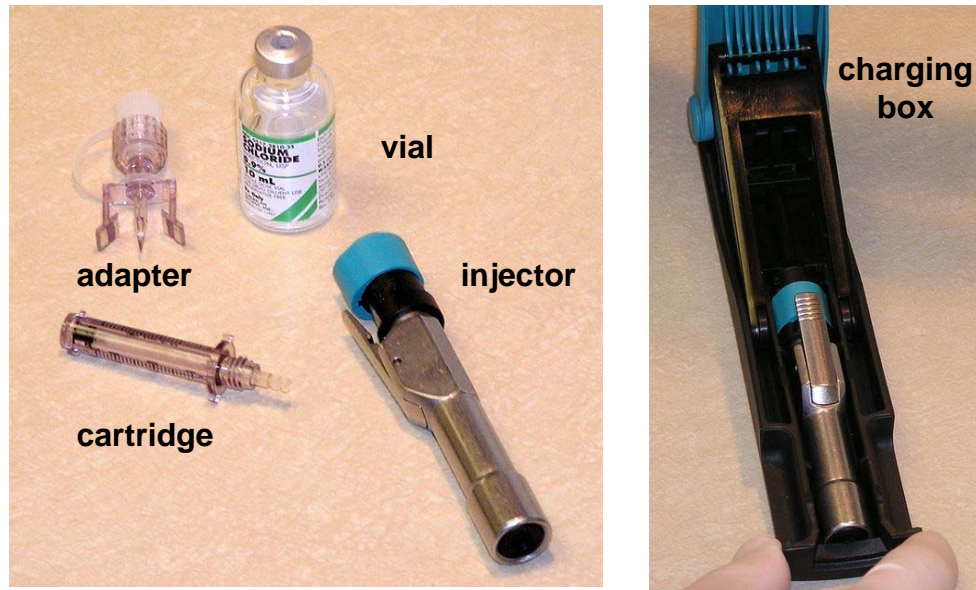
Figure A-7. (Left) The Injex30 device with a cartridge, vial adapter, and sample vial. (Right) The device is placed in the charging box to load the spring.

Injex Device	
Dose	0.05 - 0.3 or 0.05 - 0.5 mL
Weight	75 grams (Injex 30)
Power	Spring
Charge method	Charging box, manual
Lifetime	Several thousand injections
Device cost	To be determined
Part cost	Cartridge: To be determined
	Vial adapter: To be determined

Table A-8. Details on the Injex device tested in the DR.

In addition to a demonstration and oral instructions, subjects were provided with step-by-step instructions, which detail the injection process.

General design comments/suggestions by participants

Subjects mentioned several points that were pertinent to both devices. These are summarized here.

- Many people commented that the process took too much time. They were concerned mostly with the vaccine getting too warm and disrupting the cold chain. Keeping vaccine cold for as long as possible before loading the injector was important.

- Knowing that the device was still effective and within its life cycle (i.e. the spring can still discharge with full force) was important. One person suggested having a test station to shoot the device into to ensure the device would properly inject into the patient. Another suggested having a counter on the device to know how many injections were left on it.

- Fixing the device would be difficult because there are limited maintenance workers with appropriate expertise. Maintenance workers who fix the vaccine storage refrigerators would be the closest possibility. Subjects recommended that the device be designed to be thrown away rather than fixed when it requires maintenance.

- Being able to use the same device with different cartridges for IM, SC, and ID injections was very appealing. There was a suggestion to use different color cartridges for the different injection types to make it easy to improve cartridge differentiation.

- Contamination was a concern throughout the process of using a NFI.

Specific device comments by participants

Subjects were observed using the devices and were then asked questions about them. The feedback is organized by injector type and divided into steps. For each step, we

include participant comments, our observations, and recommendations based on this feedback.

Bioject

General comments by participants:

- Almost all participants commented that the injector was easy to use.

- Participants liked being able to deliver different volumes of vaccine with the device.

- A safety on the device to prevent accidental discharge was very important to many of the participants and mentioned as a necessary improvement for the device.

Aesthetics/industrial design:

Subjects were overall positive about the design of the Bioject. They thought it was child-friendly and not intimidating. The approachability of the device would help increase coverage by reducing fear of injection. A few people expressed concern that the device did not look sturdy enough and could break if dropped. Some thought that the flanges on the knob would break after being wound several thousand times.

Sterility:

Administrators and policy makers commonly asked if the device could be sterilized. While the disposable cartridges are sterile, the device is not. The manufacturer recommends cleaning only the outside surface of the device with soap and water

Placing vial adapter onto vial

Figure A-8. (Left) The vial adapter pierces the rubber skin of the vial with its plastic puncture needle. (Right) Subject placing vial adapter onto saline vial.

Observations: Participants did not have trouble placing the vial adapter onto the vial. Contamination seemed to be a concern with participants, especially since they do not wear gloves when giving injections. Participants were previously trained to hold the vial by its neck in order to maintain the cold chain and were hesitant to grip it elsewhere, for fear of making it too warm.

Participant comments:

- Keep the puncture needle of the adapter as short as possible to ensure vaccine is not wasted.

- When placing the adapter onto the vial, a user's fingers need to be quite close to the adapter head and can easily contaminate it. Several participants thought that a better design would make it easier to keep their fingers away from the head.

Recommendations: Include in training how to place adapter onto vial without making the vial warm. Enlarge the base of the adapter to enable an easy grip for the thumb and forefinger far away from the connector to the cartridge. Make the puncture needle as short as possible.
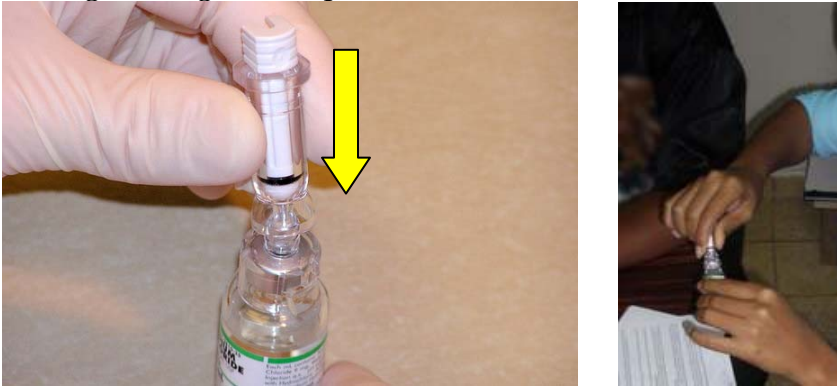
Placing cartridge on adapter

Figure A-9. (Left) The cartridge connects to the vial adapter with a press fit. (Right) A campaign worker completes the step.

Observations: Participants were all successful at placing the cartridge on the adapter, though some had difficulties with this process. The most common problem was attributable to the threads that exist on the adapter. Participants often attempted to screw the cartridge onto the adapter like a Luer lock instead of simply press fitting it on. Some had trouble placing the nozzle properly onto the adapter head and would slightly miss before aligning correctly, possibly contaminating the cartridge and/or the adapter.

Participant comments:

- It is important to maintain sterility if the vial is to be used later. Currently, the self-sealing rubber cap on a vial protects the vaccine from contamination when a needle is removed. One person suggested a similar auto-seal when each cartridge is removed from the vial adapter. If the adapter is to be kept on, then a cap is needed to ensure sterility.

- Aligning the nozzle of the cartridge onto the adapter could be made simpler.

- Keeping fingers away from the body of the vial was important so that the vial didn't warm up, but placing at the neck felt too close to the head of the adapter and could possibly contaminate it.

Recommendations: While screwing the cartridge on could take slightly more time, it may be more intuitive for users to do so. Include a cap with the adapter (researchers learned later that the manufacturer has caps for the adapters for use with the multi-dose vials).

Loading cartridge on device

Figure A-10. (Left) The cartridge, vial adapter, and vial slide into the device from the side. The flanges on the plunger should be aligned to the cartridge. The colored collar is then twisted one quarter turn to lock the cartridge in place. (Right) A nurse twists the collar of the Bioject to secure the cartridge.

Observations: Participants liked the overall simplicity of inserting the cartridge/vial into the device from the side and then locking the collar of the device. One aspect that was not clear to some was to align the flats of the plunger to the flats of the cartridge. Several people forgot to do this and then tried to force the cartridge into the device, occasionally getting it stuck.

Recommendations: Eliminating a rotational alignment of the cartridge to the device or preventing rotation of the plunger with respect to the cartridge would make this step simpler.
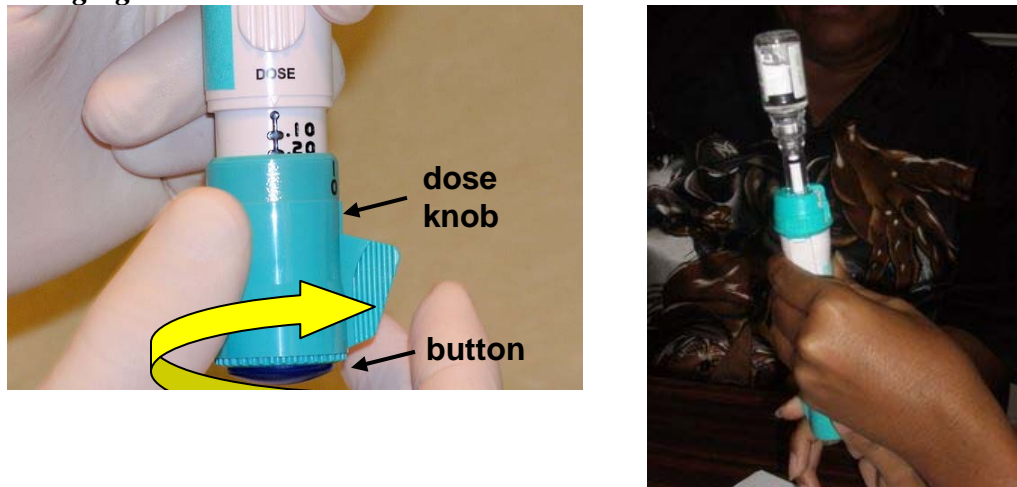
Charging device

Figure A-11. (Left) The spring within the device is charged by twisting the dose knob until it clicks. (Right) A subject charges the spring.

Observations: This step seemed to take the most time of all steps involved. All participants were able to successfully wind the device, though some did it in much more time than others (ranging from 10 – 60 seconds). No one complained about the winding being too difficult, though we had concerns that nurses doing this all day long would tire and could be at risk for repetitive stress injuries. Sometimes nurses thought they were done charging the spring and stopped twisting before the device was fully charged.

Participant comments:

- The clicking is helpful to know when the device is charged.

Recommendations: Charging the device before inserting the cartridge would allow them to keep the vials colder for longer. Enhance the sound and feel of the click. Make the button pop out more when the spring is charged.

Loading vaccine

Figure A-12. (Left) The knob is twisted in the reverse direction to load the vaccine. (Right) A campaign worker watches the markings on the device as he loads the vaccine.

Observations: Participants were all able to successfully load the saline in the vial into the cartridge. When bubbles entered the cartridge, most subjects were able to remove them after winding the knob back and forth or shaking the device. Getting the correct dosage seemed to be the key issue in this step. Participants tended to focus most on the cartridge and not on the knob, which sometimes resulted in the knob coming off completely when it was turned too much. Others focused too intently on the knob for correct dosage, which led to bubbles getting into the cartridge.

Participant comments:

- There should be a click to know that the device has loaded the correct volume of vaccine, similar to the click when the device is done charging.

- It would be nice to have volume markers on the cartridge in addition to the device.

- The volume markers on the device are too imprecise.

Recommendations: Add markings to the cartridge to keep the user's attention on the vaccine. Adjust the design of the tip of the vial adapter to reduce bubbles from adhering there. Prevent the knob from coming off the device.

Removing vial adapter

Figure A-13. (Left) The vial adapter and vial are removed by gripping the adapter and pulling away from the device. (Right) The subject grips the vial instead of the adapter to remove the vial and adapter parts. Also, note the proximity of the discharge button to the table.

Observations: This step proved to be difficult for some. Often the cartridge had been press-fit too hard and could not be pulled off easily. Some participants wanted to grab the vial instead of the vial adapter and ended up pulling off only the vial, leaving the vial adapter attached to the cartridge. Occasionally this step risked accidental discharge of the device, as most people still had the device vertically after the last step. When the two parts were separated, the hand holding the device moved quickly downward, potentially allowing the discharge button to hit the table. This sharp movement down was amplified when the parts were press fit too hard together.

Recommendations: Using a Luer lock attachment between the cartridge and the adapter and having a trigger safety on the device would ensure this step was easy and safe. Also, having a more secure connection between the vial adapter and the vial would prevent the vial and the vial adapter from separating, even if the vial instead of the adapter was gripped.
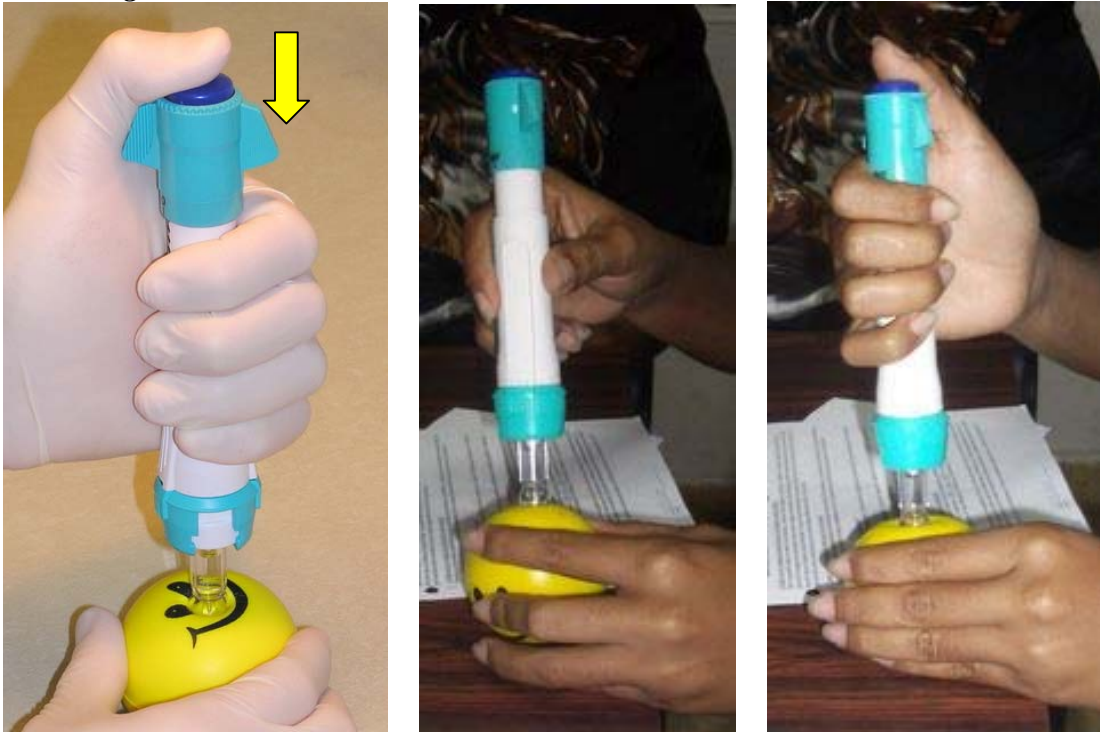
Delivering vaccine

Figure A-14. (Left) Pressing the blue button triggers the device. (Middle) For some, this grip did not come naturally, but was corrected (Right) after suggestions by the investigators.

Observations: Holding the device properly was not intuitive to some. Some held it with two hands or wanted to use the index finger to press the button. This was more common with participants with small hands. Once reminded of the correct way to hold the device, people generally understood. For people with small hands, triggering the device was a somewhat unstable process, as their hand was holding the device fairly far away from the injection side of the device, creating a large lever arm. This instability was amplified by the "patient" used for the usability testing, a yellow foam ball that tended to roll easily from under the device if it was not constrained with the participant's other hand. The noise of the discharging spring seemed to surprise several people.

Participant comments:

- There was no clear preference for trigger position. Many people liked the position of the triggering button on the device. When asked if they would want a trigger on the side, some said they would, but some said they liked it where it was.

- Having a clear cartridge was good because one could see the vaccine being delivered.

- The speed of the injection was appealing.

- This would be easier to use than NS for injecting into fidgeting patients.

Recommendations: Move the grip and trigger closer to the injection side of the device to increase stability during injection. Add an ergonomic grip to make proper gripping position obvious.

Disposal
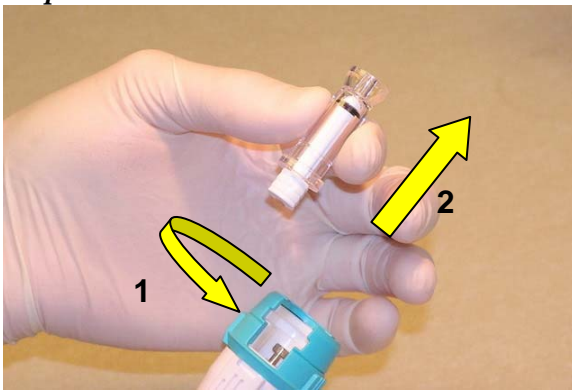
Figure A-15. The cartridge is disposed of by (1) twisting the collar and (2) removing the cartridge from the side, either by hand or by dropping it into a waste container.

Observations: Everyone was able to easily unlock the collar and unload the cartridge. People often forgot to unload the cartridge immediately, leaving a biohazard material exposed. This could be due to the subjects' excitement over completing the injection process.

Participant comments:

- The option of being able to unlock the collar and then dump the cartridge without touching it was appealing.

Recommendations: Remind users during training to remove and dispose of the cartridge immediately after injection.

Injex

General comments by participants:

- The integration of two safeties into the device was a well-liked design feature.

- The flexibility and clearness of loading different volume vaccines was appreciated.

- There were too many steps in the preparation process for an injection and too many parts of which to keep track.

- There were many opportunities to contaminate the nozzle tip during the preparation process. Minimizing the interactions with the cartridge would reduce contamination opportunities.

Aesthetics/design

Participant comments:

- The steel design was seen as very strong, sturdy, and modern. It looked like it could be easy to sterilize. While people liked this aspect of the metal design, some thought that it looked too industrial.

- Certain participants with larger hands found the device, especially the Injex 30, too small. Those with smaller hands liked the form factor.

Charging device

Figure A-16. (Left) The device is charged by loading the device in the charging box, (1) closing the lid, and then (2) opening it. (Right) Subject loading the device into the charging box.

Observations: Some people had trouble loading the device into the device charger—it was not apparent to them that it had to go in at an angle. Many people forgot to pull back the safety collar first, which prevented the device from dropping into the charger correctly. Once the device was in the charger, people tended to easily close the lid to charge the device.

Participant comments:

- Having a separate loading box and device makes it easier to lose a part

- Charging the device in the box is easy to do.

Recommendations: Revise the charging box design to make it more intuitive to load.

Placing vial adapter onto vial

Figure A-17. The vial adapter pierces the vial with a plastic puncture needle. The adapter arms lock the adapter atop the vial.

Observations: This step was generally easy for everyone. The way that the cap of the adapter was attached to the head was a bit difficult for participants to take on and off because when they unscrewed the cap, the cap leash wanted to twist as well.

Participant comments:

- The needle should be shorter to ensure there is no vaccine waste

Recommendations: Eliminate the cap leash. Make the vial adapter self-sealing. Make the puncture needle as short as possible.
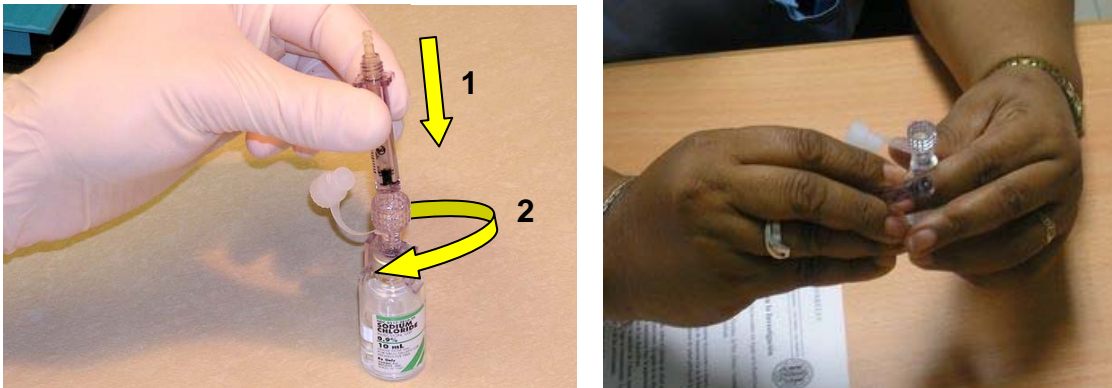
Placing cartridge on adapter

Figure A-18. (Left) The cartridge is loaded onto the adapter by (1) placing it atop the adapter and (2) twisting it into place. (Right) A subject prepares to load the cartridge on the adapter.

Observations: About half the time, participants wanted to put the plunger side of the cartridge rather than the nozzle side onto the vial adapter. Once the correct side was determined, screwing on the cartridge was generally successful. Sometimes the cartridge was a little loose.

Participant comments:

- Contamination in this step was a large concern if the cartridge was misaligned and when the cap was unscrewed

Recommendations: Make the cartridge have a clearer front and back to prevent confusion when placing the cartridge on the adapter.
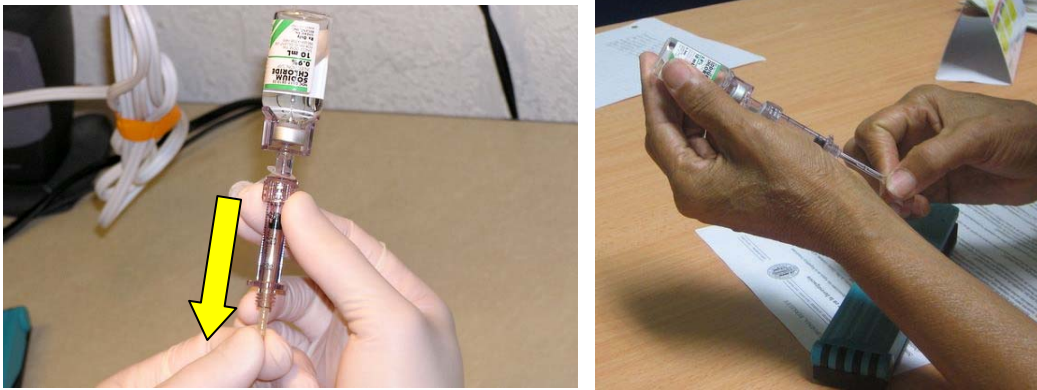
Loading vaccine

Figure A-19. (Left) Vaccine is loaded into the cartridge manually by pulling the plunger back while the vial is upside down. (Right) A HCW loads saline into the cartridge.

Observations: Subjects completed this step successfully. Some had difficulties getting all of the air bubbles out. The volume gradations on the sides were easy to read.

Participant comments:

- Some were concerned that when they withdrew the plunger and then pushed it back in to remove air bubbles, then pulled the plunger back out, that they were contaminating the inner walls of the cartridge

- A stop on the plunger would be useful so one cannot accidentally pull the plunger out all the way

- Putting a cover on the plunger stem would prevent contamination when loading vaccine.

- This step should be faster.

Recommendations: Use a more hydrophilic coating on the cartridge to reduce bubbles, if it were compatible with the vaccines.
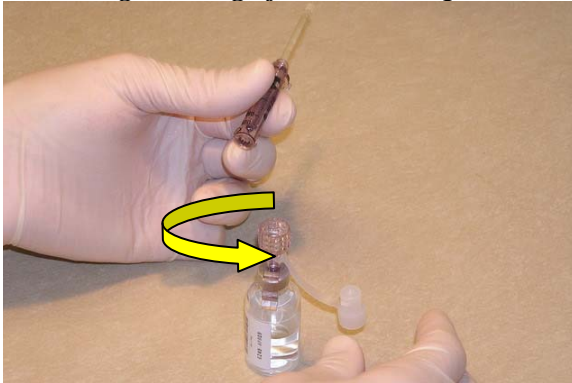
Removing cartridge from vial adapter

Figure A-20. The drug-loaded cartridge is twisted off to remove it from the vial adapter.

Observations: This step was fairly straight forward for most participants, though there was concern of contamination when unwinding. Participants for the most part also left the vial uncapped, leaving the vial at risk for contamination.

Recommendations: Modify the cartridge design to have a protective shield to prevent contact with the cartridge nozzle.

Loading cartridge on device
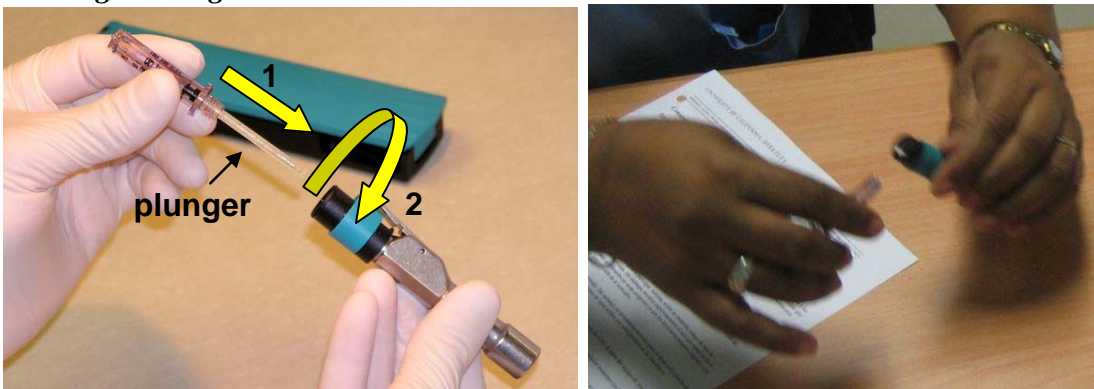
Figure A-21. (Left) The cartridge is loaded by (1) inserting the plunger end down the barrel of the device and then (2) twisting the cartridge firmly until it locks in place. (Right) A subject initially tries to load the cartridge backwards.

Observations: Many people tried to insert the nozzle end of the cartridge into the device. Once the plunger was inserted correctly, people were able to twist on the cartridge. Often participants did not twist the device on tightly enough, and the safety was not disabled, preventing injection.

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Recommendations: Give the user a signal such as a click that the cartridge is twisted on enough. Use a color code for the ends of the vial adapter, plunger, and device to make matching ends easier.

Delivering vaccine
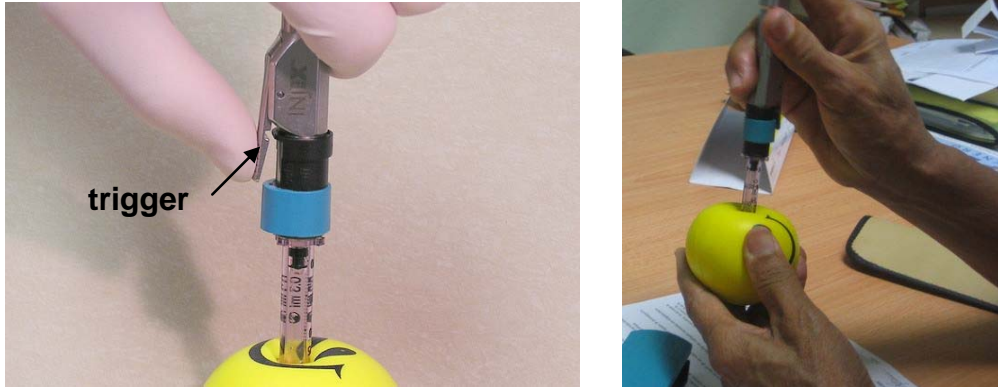
Figure A-22. (Left) The injection is completed by pushing down the safety collar and pressing the trigger of the device. (Right) The HCW initially holds the device the wrong way. Later she correctly injected the ball.

Observations: Gripping the device correctly was not intuitive for many people. Because of this, it was difficult for them to press down the collar and then inject. Once showed how to do this, the participants were able to inject. The noise of the discharging spring seemed to surprise several people.

Recommendations: Encase the metal device with a plastic grip to assist users to hold it correctly. Replace the moveable safety collar with a pressable button.

Disposal

Figure A-23. The cartridge is removed by untwisting it from the device.

Observations: Generally there were few issues with disposal of the cartridge, though it was difficult for people to do without touching the nozzle tip. Sometime people 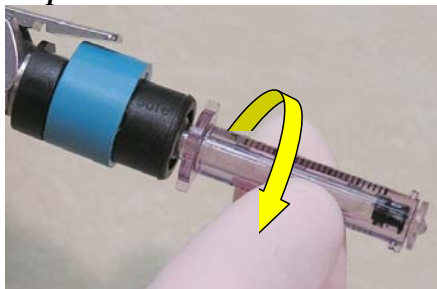wanted to put the device back in the charger when done, but we discouraged them from doing this so that the spring would be left unloaded.

Recommendations: Add a protective shield to the cartridge.

Summary of Device Requirements

Based on the feedback on the concept of NFI and on devices, we determined the following design and performance requirements for a device to be used in the DR:

Design	
	Simple preparation and injection
	Integrated safety
	Disposable cartridge
	Maintenance free
	Non-electrically powered (i.e. spring)

Performance	
	Proven clinical effectiveness
	Cost competitive with NS injection
	Time competitive with NS injection
	Compatible with current infrastructure
	Able to deliver intradermal, subcutaneous, and intramuscular injections

Table A-9. Summary of design and performance requirements for a NFI device for use in the DR immunization system

A suitable device for the DR must be simple to prepare and inject, maintain the cold chain, and also prevent contamination. A safety on the device will prevent its accidental discharge during the preparation. Any NFI device must have a disposable cartridge. Since repair will be difficult to impossible, the device must be maintenance-free.

Because vaccination clinics may have limited resources, the NFI device must be powered without the use of electricity or compressed gas.

With regards to performance, the NFI device must be proven to be clinically effective with all vaccines planned to be delivered with it. The device must be cost competitive with NS injections and also must take the same or shorter amount of time to prepare and inject a vaccine. NFI supplies must fit into the current distribution infrastructure. Lastly, the device must have the flexibility to deliver vaccines to the necessary injection depths.

OBJECTIVE 3: PROPOSE AN IMPLEMENTATION PLAN FOR NEEDLE-FREE INJECTION ADOPTION IN THE DOMINICAN REPUBLIC

In this section, we discuss important issues and considerations for the adoption of NFI and propose an implementation plan. First, we discuss the process that would occur prior to the decision being made to implement NFI or any other technology into the immunization program in the DR. Second, we propose steps that could be taken to ensure a successful implementation of NFI. Lastly, we describe some issues that should be considered in the years following an implementation of NFI to address the long-term sustainability of the technology.

Decision

Prior to implementation of NFI, efforts should be made to ensure that NFI properly address the issues and design criteria listed in Table A-5 and Table A-9, respectively. Furthermore, in order to implement a new technology in the immunization system, that technology must gain the endorsement and support from the following government and non-government organizations.

Expanded Programme on Immunization (PAI)

The national director of PAI is its key decision-maker and would need to be in support of NFI before it could be implemented. The director would review all available literature on the technology and would seek input from members of his staff, including but not limited

to technicians, the cold chain coordinator and the training coordinator. The director

would also likely seek input from immunization providers and the ICC.

Ministry of Health and Social Assistance (SESPAS)

If the PAI director decided to support implementation of NFI, he would present his

opinion to the SESPAS Secretary of Collective Health, whose office oversees PAI.

SESPAS technicians would then analyze the technology to see if it would provide

improved quality of care at manageable costs. The Secretary would obtain feedback from

these technicians and also decision makers at PAI and ICC. If the Secretary of Collective

Health decides to endorse NFI, he would present the technology to the Secretary of

Health, who would make the final decision about whether or not to implement NFI.

Interagency Coordination Committee (ICC)

Before PAI and SESPAS would decide to implement a new injection technology, they

would most likely require an endorsement from ICC, particularly PAHO and UNICEF.

These organizations in turn would look to their own research efforts, published literature,

and regulatory agencies such as the US Food and Drug Administration (FDA) to approve

devices as safe and effective. Upon completion of evaluation studies, the WHO and/or

UNICEF would likely draft guidelines that explains the proper usage and disposal of the

device as they have done with AD syringes (18).

Implementation

The successful implementation of a new technology requires careful consideration of

how it will fit into the existing infrastructure and what changes, if any, will need to be

made to accommodate the technology. The following is a list of the most important implementation considerations raised by our interview subjects.

Financial burden

While we expect recurring annual costs of NFI to be similar to current immunization costs, implementation of NFI will require additional funding. Support for the introduction of NFI could be provided from various types of funding arrangements. One such possibility is consulting with the ICC to coordinate donor funds for the introduction of the new technology that gradually decrease over a set amount of time. In this way, the DR government would become more responsible for funding devices each year and eventually fully support the technology. This strategy is beneficial because it allows the country to introduce the new technology while gradually securing funding in the national budget. This strategy was used for the introduction of the pentavalent vaccine, for which the original funding was provided by the government of Japan (see case study on page 169). A second method of funding an NFI implementation is manufacturer subsidy such that the price per device is initially reduced to affordable levels for SESPAS and increases with time. In this case the manufacturer bears the initial financial burden but secures a large market. A third option for funding these devices is SESPAS paying for the entire cost. A summary of the benefits and challenges of each of these structures is shown in Table A-10.

Initial Funding Source	Benefits for the DR	Challenges for the DR
International agency	- Allows DR time to secure funds for new technology - Donor pays for costs of introduction - Can use any manufacturer	- Need to find an agency to donate the funds - Lose some autonomy in coordinating introduction
Manufacturer	- Allows DR time to secure funds for new technology - Manufacturer pays for costs of introduction	- Need to find a manufacturer to donate the funds - Will likely need to commit to purchasing from same manufacturer for many years
DR government	- Complete autonomy in coordinating introduction - Can use any manufacturer	Need to immediately find sufficient funds in budget

Table A-10. Possible structures for initial funding of NFI

Device procurement

Assuming that PAHO has already recommended using NFI for immunizations, it already will be negotiating NFI pricing through the Revolving Fund. Calculating the exact number of devices needed for the entire country for use in routine immunization settings will require a survey of the immunization personnel simultaneously working at each facility and the number of injections given per clinic. Given that 1250 immunization posts exist in the DR, if each immunization facility has an average of two nurses giving immunizations at the same time, then PAI will need at least 2500 functional NFI devices at all times.

Distribution and storage

Distribution of NFI cartridges should be essentially the same as for needles and syringes. New NFI devices will be distributed on a quarterly or as-needed basis. Additionally, distribution of yellow biohazard sharps disposal boxes will be reduced because NFI injections do not generate as much biohazard sharps material as do NS. On the contrary, the distribution of red biohazard bags will be increased as NFI cartridges are still

considered biohazard waste (though not sharps waste). Since the current vaccine vials and other supplies except for NS and disposal materials will not change, the rest of the distribution system should be identical to the current system.

Cold chain management

In an ideal NFI implementation, there would be no change in the current cold chain management. However, there would need to be efficient procedures that minimized the time that the vaccine is exposed to ambient air during an injection procedure. As mentioned above in the Device Feedback section on page 134, there are multiple design and procedural improvements that will reduce the time the vial is exposed to air as well as clinician interaction with the vial.

NFI training

From our conversations with nurses and training personnel in PAI provincial offices, we learned that sufficient training is necessary to ensure a successful implementation of the technology. Both nurses and doctors expressed concerns with large transitions because they have not proceeded smoothly unless appropriate training and education were provided. One vaccine nurse already participating in a CDC-sponsored NFI study said that she received one week of training on device usage. Many subjects felt two to five days is to the amount of time necessary to appropriately train the nurses with NFI devices. Because there is already a regular training system in place, both nurses and trainers suggested that NFI will easily fit into existing training system.

Waste management

The management of NFI waste will be considerably different than NS waste management. First, there will be less risk of accidental disease transmission to the janitorial staff that handles biohazard waste from the immunization clinics because the waste will no longer be sharp. Next, the incinerators will not need to run as hot because the plastic-molded cartridges will melt at a much lower temperature than the steel needles (~500° C vs. 1000° C). This will slow the rate of gasoline consumption, making tanks last longer and reduce air pollution to the environment (assuming that non-PVC plastic is used for the cartridges). Third, the amount of biohazard waste will be smaller because typical NFI cartridges are smaller than conventional needles and syringes. Most interview subjects agreed that NFI implementation would have a positive effect on the safety and efficacy of waste disposal.

Patient education

Every comment we received regarding patient education stressed that the public is properly educated about the benefits of the new technology, how it works, and its efficacy. One technical analyst at SESPAS said that if people are not educated and they feel that the HCW is doing something new or incorrectly that the family will leave and go to another hospital. PAI and SESPAS have had much experience with publicizing vaccination campaigns and other initiatives and thus, given the appropriate resources, should be able to properly educate the public about NFI.

Use of NFI in campaigns

We commonly heard from our interview subjects that NFI devices should be first introduced into the clinic to ensure health workers are properly trained in a controlled environment before using the devices in campaigns. One possible concern was the training of campaign workers that do not routinely work with NFI devices. These workers do not normally give injections, and the amount of time and resources it would take to train them on NFI may be a barrier. According to a senior official at the central PAI office, as many as 10,000 immunization personnel work during campaigns. This would require the procurement of a large number of devices. The number of injections per hour is also generally much greater during campaigns than during routine practice so it is very important that the speed of the device be similar or better than NS.

We also received feedback that it would be best if immunization workers and clinicians used the same technology for all vaccines and all situations. Reduced waste with NFI would reduce work for overloaded incinerators during campaigns. Another advantage mentioned for NFI use during campaigns is the ability to carry a smaller biohazard bag instead of a biohazard box. Lastly, reduced needle anxiety could increase coverage.

Sustainability

It is important that a sustainability plan is considered and that checkpoints are in place to ensure continued success. The following is a list of the most important sustainability considerations raised by our interview subjects.

Long-term funding

Donor funding of NFI will likely only last for the first few years of an NFI implementation. After that period it is essential that the DR government can secure sufficient funding to maintain the new technology in the immunization program. A plan for this long-term national funding should be determined prior to implementation. The vaccine law that is now being considered should make it easier to secure funds for vaccines and related supplies.

Quality control

Training for the NFI must be included in the national and local training programs. It is important to ensure that the new technology is being used properly and that both nurses and patients understand the benefits of NFI. There should also be a mechanism in place for nurses to provide feedback on the use and training of NFI so that necessary modifications to the training or the device itself can be made.

Maintenance

If an NFI device ever breaks in a clinic, then there should be a backup NFI device onsite to ensure continued availability of vaccines. It might be necessary to have a limited supply of conventional needles in case all available devices fail. A constant use of the device at its introduction will reinforce the device integration into the system.

Follow-up studies

Multiple follow-up studies should be performed to gauge the effectiveness of NFI. First, a study should be done to determine whether NFI has decreased the rate of disease

transmission from unsafe injections. Second, a study should be done to determine the effect of NFI on the waste management system. Third, a study should be done on the perception of NFI amongst nurses and patients. Without sufficient acceptance by the users of the technology, it will be very difficult for the technology to be sustainable, as was the case with AD syringes (see case study on page 169).

CASE STUDY: PENTAVALENT VACCINE AND AUTO-DISABLE SYRINGES

Overview

While considering the adoption of needle-free injection in the DR it is important to consider the successes and failures of relevant recent technology adoptions. The most relevant example that we encountered is the introduction of the pentavalent vaccine and the associated introduction of AD syringes into the DR's immunization program in 2001. The pentavalent vaccine is a combination vaccine that elicits immunity against five diseases: diphtheria, tetanus, pertussis, hepatitis B, and Haemophilus influenzae type b (Hib). Prior to the introduction of the pentavalent vaccine, the diphtheria, tetanus, and pertussis vaccines were delivered together in DTP, the hepatitis B vaccine was delivered separately, and the Hib vaccine was not in standard use. AD syringes are similar in appearance and function to standard disposable syringes except that after the plunger has been pulled back to load the vaccine and pressed in to delivery the vaccine into the body, the plunger cannot be pulled back again. This mechanism prevents the reuse of AD syringes and is designed to improve injection safety. The pentavalent vaccine has been heralded as a huge success, greatly reducing the number of incidences of meningitis caused by Hib and also reducing the number of injections given per child. Conversely, AD syringes have not been widely accepted by vaccination nurses and their supply and distribution have been discontinued by the DR's PAI headquarters.

Decision

Hib/pentavalent vaccine

In the last 10 years, many developing countries have begun to introduce the Hib vaccine for routine use in their public health immunization schedules. The introduction of a new vaccine into a national immunization program depends, in part, on demonstrating that the local burden of disease warrants the cost of vaccination. This is particularly true for the Hib vaccine because the vaccine itself is far more expensive than most other common vaccines (US$3.95/dose vs. US$0.16/dose for DTP).

Following established protocols for estimating the local burden of Hib-related diseases (19-21), Gomez *et al.* (19) performed a PAHO and USAID funded population-based surveillance in the DR in 1998 to determine the incidence rates of bacterial meningitis caused by Hib and by other pathogens. The incidence of Hib meningitis was determined to be 13 cases per 100,000 children less than five years of age, which motivated policy makers at PAI and SESPAS to seek means of introducing the Hib vaccine into routine use in the DR. Around this same time, another study in the DR, funded by USAID, was attempting to demonstrate the efficiency of a more economical regimen of the Hib vaccine that used a reduced dose per patient (22). Although the results of this study indicated that a lower dose of vaccine (as low as one-third) was able to provide adequate protection against Hib meningitis, PAHO was not willing to endorse a reduced dose regimen without numerous additional investigations.

In 2000, the government of Japan and the Japan International Cooperation Agency (JICA) agreed to help fund the introduction of the standard-dose Hib vaccine in the DR for the first five years to begin in 2001, a total investment of greater than US$10 million. The Hib vaccine was to be delivered as part of the recently developed pentavalent vaccine, which had the additional advantage of reducing the number of injections given to children below one year of age. In addition to PAI and SESPAS, representatives of JICA, USAID, PAHO and UNICEF in the DR were involved in the decision-making process. UNICEF was contracted to coordinate the donation between JICA, PAI, and PAHO's Revolving Fund, which would supply the vaccine. In June of 2000, the President of the DR traveled to Japan to formalize the agreement with Japanese officials, which included a commitment by SESPAS to continue funding for the vaccine after the contract was over.

Auto-disable syringe

As early as 1986, WHO had requested the development of a single-use disposal syringe, called an AD syringe (23). If the syringe was made with a needle permanently attached, the AD mechanism would eliminate the reuse of both needles and syringes. The results of multiple field studies with AD syringes, including ones in Pakistan (24) and Indonesia (25), indicated that AD syringes were well accepted by vaccinators and did not require greater training than conventional syringes.

The recognition of the great burden of disease caused by unsafe injections and the demonstrated ability of AD syringes to eliminate the reuse of needles led WHO and UNICEF to publish a joint statement in 1999 endorsing the worldwide use of AD

syringes for all vaccination injections (26). In this statement it was announced that UNICEF program funds could no longer be used to procure standard disposable syringes and that, by the end of 2003, all countries should use only AD syringes for immunization. Proper training, supervision, and waste management were also stated as requirements to ensure safe injection practices.

JICA, PAHO and UNICEF recognized the transition in the DR to the pentavalent vaccine in 2001 as an opportunity to also introduce AD syringes into the national immunization program. They decided to supply AD syringes with the donation of vaccine, requiring the vaccination nurses in the DR to use AD syringes to inject the pentavalent vaccine.

Implementation

Funding and procurement

According to a senior PAI official, PAI purchased an initial supply (160,000 doses) of pentavalent vaccine in 2000 before the contract with Japan began. However, for the first full year of the contract (2001), Japan provided 100% of the required funds for the vaccines and associated supplies and support. The rollout of the new vaccine was done first in Santo Domingo and then extended to rural areas. The first Japan-funded shipment of vaccine came with AD syringes. As per the agreement between the two countries, Japan's commitment decreased incrementally 20% each year with SESPAS covering the difference until SESPAS was providing 100% of the funds by the sixth and final year of

the contract (2006). These funds included the purchase of both vaccines and syringes from PAHO's Revolving Fund and were coordinated by UNICEF.

Training and patient education

The training for the rollout of pentavalent vaccine took four to five months. In this training, the nurses were explained why the pentavalent vaccine was being introduced and what the main benefits of the pentavalent vaccine were. The nurses were taught the importance of explaining to their patients why and how they would be receiving fewer injections but would actually be receiving more vaccines. According to policymakers and administrators, this emphasis was crucial to ensure acceptance of the new vaccine by both nurses and patients. The training also included a refresher course to emphasize proper vaccination procedures.

According to a senior PAI official, the training for the pentavalent vaccine included very little instruction on the proper use of AD syringes, despite the fact that AD syringes were to be used with the pentavalent vaccine and that they were being introduced in the DR for the first time. This official reported that, in fact, the primary means of training on AD syringes was the distribution of the instruction pamphlet that came with the syringes. However, in some areas of the country, including Peravia, nurses we spoke with indicated that they had been trained on how to use the AD syringes.

Distribution and storage

The pentavalent vaccine vials and AD syringes were stored and distributed in the same manner than other vaccines and conventional needles and syringes were previously.

Sustainability

Hib/pentavalent vaccine

The pentavalent vaccine has been a large success in the DR. In regards to the incidences of Hib meningitis, the notion of the "before pentavalent era" and the "after pentavalent era" is often mentioned. Since the introduction of the pentavalent vaccine the number of cases of Hib meningitis has decreased from approximately 300 per year to less than 10 (27). In addition, nurses and patients have accepted the new vaccine and appreciate the reduced number of injections.

The pentavalent vaccine is now being paid for entirely by SESPAS as the contract with Japan expired. There was a brief lapse of availability of the pentavalent vaccine at the beginning of 2007 but this has been attributed to a supply issue with the vaccine manufacturer and not to management issues with PAI (28). The continued financial support of the pentavalent and all other vaccines should be guaranteed with the passage of the vaccine law which is now being considered by the ICC, PAI and the President of the DR.

Auto-disable syringe

Unlike the pentavalent vaccine, the associated AD syringe was not well accepted by nurses. Needle reuse is generally not a current problem in the DR because of a constant supply of needles and syringes so many interview subjects felt that technology to prevent needle re-use was not necessary. In the DR, a recent study from 2007 reported no

evidence of NS reuse in any of the immunization clinics or hospitals they surveyed throughout the country (17).

Many of our subjects were displeased with the practical use of AD syringes. If a bubble was accidentally drawn into an AD syringe, it could not be pushed out without engaging the mechanism that locks the plunger after a single injection. When this occurs, the disabled syringe must be disposed along with the attached needle. This issue is made worse because there was no formalized training procedure for AD syringe use and the pentavalent vaccine is provided in a single-dose vial increasing the possibility of pushing the point of the needle through all of the liquid while withdrawing the vaccine. There were some locations where nurses reported that they did like using AD syringes because of the improvement in safety but most nurses disliked using them.

After two deliveries of AD syringes along with the pentavalent vaccine, PAI requested that UNICEF discontinue providing AD syringes. Subsequently, conventional disposable syringes were supplied. With SESPAS now funding 100% of the pentavalent vaccine and associated supplies, AD syringes are not being procured by PAI though there are some regions and areas that are still using their last remaining supplies of AD syringes. The decision by PAI to halt the distribution of AD syringes was based on the following information: 1) AD syringes cost 80% more than conventional disposable syringes; 2) AD syringes were not well accepted by nurses; 3) AD syringes only addressed unsafe injections related to needle reuse, which is reportedly not a problem in the DR, but did not address issues with accidental needle-pricks or biohazard sharps waste disposal.

Key Takeaways

From this case study, we can take away the following about the adoption of new technology such as NFI technology:

1. Prior to making a decision to implement a potential new technology, it is critical to consider that the technology is a solution to a current significant problem in the location in which the technology is to be implemented. For example, a local burden of disease study is essential for the introduction of new vaccination technology, particularly one that will raise costs. In this case study, significant additional funds were required for introduction of the pentavalent vaccine but the local burden of Hib meningitis warranted the investment. In the case of AD syringes, although it had been established that AD syringes could greatly alleviate unsafe injections from needle reuse, needle reuse did not appear to be a significant problem in the DR.

2. A proper training process must occur prior to implementation of the new technology. It is possible that the reason that the nurses in the DR had difficulty using AD syringes and did not like to use them is because they did not receive proper training. From our interviews, it seems that the training associated with the introduction of the pentavalent vaccine did not include much guidance on the proper use of AD syringes. Interview subjects who had been trained on the use of AD syringes had an easier time using them and, in many cases, actually liked using them. With proper training, studies have shown nurse acceptance of AD syringes in other countries in pilot studies (24, 25, 29) and even in nationwide implementation (30).

DISCUSSION OF ALTERNATIVE APROACHES

Other approaches to NFI exist for improving injection safety, and these should be considered as alternatives to NFI. These fall broadly into the following categories: new injection supplies/technology, increased injection safety training and monitoring, improved waste management, increased vaccine coverage of HCWs, and focused efforts on improving therapeutic injection safety. Data from Moro *et al*. (17) on needle-stick injuries is very revealing on where needle safety practices could be improved in the DR.

Injection safety training for HCWs in immunization clinics for injection and waste management

Increased training of both immunization nurses and campaign workers could help improve needle safety and reduce the rates of unsafe sharps. As mentioned previously, Moro et al. found that injection safety was a significant problem at immunization clinics. In immunization clinics, 10% of HCWs reported a sharps injury in the 12 previous months before the study (17). While the existence of needles will always have an inherent risk of sharps sticks, better compliance with injection safety practices could reduce this risk.

Supplies do not appear to be a problem with immunization clinics, as yellow safety boxes are supplied to all clinics and needle reuse is virtually non-existent. Biosafety guidelines were developed by PAI in 2001 for safe injection practices, and these guidelines could be reinforced in all workers. For immunization workers, training takes place at least two times a year, and improved safety practices could be reinforced.

Benefits: Small reduction in accidental needle pricks, training could be included in standard training of HCWs, low additional cost.

Challenges: Needles still present a hazard. Given training already occurs, it is unclear if more training will have a large impact.

Increase training for HCWs in therapeutic clinics and hospitals

Statistics reported by Moro *et al.* for hospitals (outside of the immunization program) were worse than inside the immunization clinics. Of the HCWs interviewed, 45% of them had a sharps stick in the previous year. Yellow biohazard boxes are not supplied to hospitals (17). HCWs improvise and use alternative puncture-proof containers for sharps disposal. Sharps were observed to be discarded in these containers 94% of the time after an injection, but 92% of regular waste containers near the injection area were observed to contain sharps. Lastly, 70% of HCWs at the hospitals surveyed reported never being trained in safe injection practices (17). While this study only looked at two hospitals and may not be entirely indicative of the hospital system as a whole, these numbers suggest a problematic trend.

Providing training to these HCWs and waste management workers would have the potential of greatly improving injection safety in the country. The international health organizations USAID, JICA, and UNICEF have all been working with individual health regions to improve waste management, which may positively impact these statistics.

Benefits: Biosafety performance at hospitals underperforms immunization clinics (17) and behavioral changes here could have a large impact. Donor organizations have

identified this area as part of their mission in the DR and are willing to fund efforts for biosafety improvements from a behavioral perspective.

Challenges: The immunization program has a well organized top-down management structure with an efficient supply chain and existing training programs, making education and changes to the program readily implementable. In our understanding, this training structure is non-existent in the hospital system as a whole and changes would need to be addressed on a hospital by hospital basis, making the job time and resource intensive.

Increased training for waste disposal workers

Needle-stick injuries of waste management workers have not been well documented in the DR, though could be high. Moro *et al*. documented inadequate waste management, particularly in hospitals and therapeutic clinics (17). Including janitorial workers in sharps safety training would likely have a greater impact than just training HCWs.

Benefits: Training waste management workers could reduce needle-stick injuries among a high-risk population.

Challenges: Inclusion of waste management workers in safety training would require high effort, as they are employed by individual hospitals and minimal training of this group currently exists.

Injection supplies/technology

Gloves: Currently, gloves are not used by HCWs in immunization clinics in the DR. People interviewed said that gloves are not used because of their extra cost and the impression that nurses would find them uncomfortably hot in their clinics, most of which do not have air conditioning, and on campaigns.

Benefits: Glove use would reduce accidental contact with biological fluids but does little for preventing accidental needle pricks.

Challenges: Training would need to be done to educate nurses on the benefits of disposable gloves. They would need to be adequately convinced that gloves are worth their discomfort in order for their use to be sustainable. PAI would have a long term increased cost due to additional supplies and increased logistical challenges in distribution and storage.

Safety syringes: Safety syringes, which have a retractable needle or an extendable collar to shield the needle, would greatly decrease accidental needle pricks, both to clinicians and waste management workers.

Benefits: Reduction in accidental needle pricks.

Challenges: Safety syringes are not in country-wide use in any national immunization program to our knowledge. Safety syringes have a higher cost and are larger, making incineration more challenging. Initial training would need to be done to introduce their use, and PAI would have an increased long-term cost of safety syringe purchase.

Increase coverage of Hepatitis B among HCWs

While infant coverage rate of Hepatitis B vaccine (HBV) is reported by PAI to be 87%, coverage among HCWs is much lower—65% of immunization clinic HCWs and 55% of hospital HCWs have completed all three doses of the vaccine (17). Running a campaign to increase coverage of HBV among HCWs would reduce transmission of Hepatitis B in the case of needle-stick injuries.

Benefits: Increased protection of HCWs in immunization clinics and hospitals, less significant long-term costs.

Challenges: Cost of running a campaign for HCWs, continued push to keep HCWs vaccinated with staff turnover, does not address other diseases communicable by blood, such as HIV and Hepatitis C.

Criteria for making a decision

When weighing the options, we recommend the following criteria be considered:

Cost: Overall cost (short and long term) is of critical importance to the immunization program. Any large increase will need to be supported by donor organizations or by SESPAS and the congress.

Impact: Any initiative worked on will need to have a large impact to justify the capital put up for the project

Feasibility: The capabilities of PAI and the support of donor organizations must be considered.

Sustainability: The best initiatives must have a clear path to future sustainability.

Need: The solution must have a clear need.

	Increased immunization HCW training	Glove use	Safety syringes	Vaccinate HCWs	Increased waste management worker training	Increased hospital HCW training	NFI
Cost	Low	High	high	low	high	high	Medium
Impact	Low	Medium	medium	medium	high	high	High
Feasibility	High	Medium	medium	medium	medium	medium	Medium
Sustainability	High	Medium	high	medium	medium	medium	Medium
Need	medium	Medium	medium	high	high	high	High

Table A-11. Comparison of alternate technologies vs. NFI when looking at decision criteria.

When comparing against these criteria, several options stand out as promising solutions to the challenges and needs of the DR PAI. Devoting increased attention to waste management education and training scores high across all criteria. Increased training in

non-immunization aspects of the public health system also rates high, though is outside the scope of PAI. Lastly, NFI is strong in all criteria areas, showing it to be a promising candidate for addressing challenges of PAI. These solutions are not exhaustive nor are they exclusive—several of them could be done concurrently to reduce disease transmission due to unsafe injections.

CONCLUDING REMARKS

Multiple studies have shown that unsafe injections with conventional NS in developing countries are a major contributing factor to the transmission of blood-borne disease. This issue has motivated the development of alternative means of injection with improved safety. NFI is one alternative that is safer than conventional needles simply because it reduces biohazard sharps. However, additional analysis is required to determine the effectiveness, affordability, and sustainability of NFI for developing world settings. Furthermore, before NFI technology could be implemented in a specific country, it is necessary to consider if the technology is appropriate for that country and whether it is the best option amongst all available alternatives. As we learned with the AD syringe, it is extremely important to understand the local context and local needs to accurately evaluate the appropriateness of any solution.

In this study, we sought to determine if NFI is appropriate for the DR and how the technology could best be improved to meet the needs and concerns of that country. First, we learned that unsafe injections do occur in the DR, mostly in the form of accidental needle pricks and inappropriate waste management. NFI was thought to have the potential to improve vaccine coverage due to lack of needles while reducing disease transmission resulting from unsafe injections. To determine if these effects are significant enough to warrant the adoption of new injection technology, a formal study would need to be performed to quantify increased coverage and the disease burden resulting from unsafe injections. Second, we have identified issues that would need to be considered/addressed to satisfy the concerns of the policy makers and show that NFI is a

feasible upgrade for the immunization system. Third, we have laid out recommendations on how the design of NFI devices could be improved to fulfill the requirements of the HCWs that would be using it. Lastly, we have made some recommendations on how NFI could ultimately be implemented in the DR.

Based on our interviews and observations, we believe that NFI is a long term solution to injection safety challenges facing the DR. An immediate and significant reduction of needle use will provide a safer environment for patients and HCWs and may increase coverage due to the general human fear of needles. NFI will also result in less precarious waste disposal because of the reduction in biohazard sharps waste, which could further reduce the transmission of blood-borne disease. We recommend taking the following actions to determine if NFI is the best course of action for improving injection safety in the DR:

Issue	Action
Need	Formal study assessing potential increased coverage with NFI and burden of disease caused by unsafe injections
Effectiveness	Clinical trials with NFI in the DR or similar settings
Affordability	Detailed cost model of NFI
Sustainability	Pilot introduction of NFI in the DR to assess use/acceptance

Table A-12. Further actions that should be taken before the DR would decide whether to approve NFI for immunizations.

The research presented here is part of a global effort focused on identifying alternative methods for vaccine and drug delivery. Numerous NFI studies in other countries have shown similar results and interpretation to those shown here. Taken together, we are encouraged to see that many governments are committed to improving health care, starting with preventative measures such as immunization technology, and that the global

health community is progressing towards a consensus view that improving injection safety is feasible in developing world settings.

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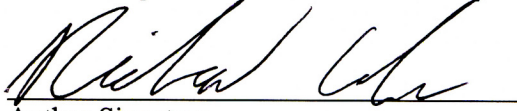
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