

Highly Efficient, Large Volume Flow Electroporation

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Electroporation is widely used to transfect and load cells with various molecules. Traditional electroporation using a static mode is typically restricted to volumes less than 1 mL, which limits its use in clinical and industrial bioprocessing applications. Here we report efficient, large volume transfection results by using a scalable-volume electroporation system. Suspended (Jurkat) and adherent cells (10T1/2 and Huh-7) were tested. A large macromolecule, FITC-conjugated dextran (MW=500 kD) was used to measure cell uptake, while a plasmid carrying the gene coding for enhanced green fluorescence protein (eGFP) was used to quantitate the flow electrotransfection efficiency as determined by flow cytometry. The flow electroloading efficiency of FITC-dextran was >90%, while the cell viability was highly maintained (>90%). High flow electrotransfection efficiency (up to 75%) and cell viability (up to 90%) were obtained with processing volumes ranging from 1.5 to 50 mL. No significant difference of electrotransfection efficiency was observed between flow and static electrotransfection. When 50 mL of cell volume was processed and samples collected at different time points during electroporation, the transgene expression and cell viability results were identical. We also demonstrated that DNA plasmid containing EBNA1-OriP elements from Epstein-Barr virus were more efficient in transgene expression than standard plasmid without the elements (at least 500 to 1000-fold increase in expression level). Finally, to examine the feasibility of utilizing flow electrotransfected cells as a gene delivery vehicle, 10T1/2 cells were transfected with a DNA plasmid containing the gene coding for mIL12. mIL12 transfected cells were injected subcutaneously into mice, and produced functional mIL12, as demonstrated by anti-angiogenic activity. This is the first demonstration of efficient, large volume, flow electroporation and the *in vivo* efficacy of flow electrotransfected cells. This technology may be useful for clinical gene therapy and large-scale bioprocesses.

Key words: flow-electroporation, transfection, large-volume

Introduction

The recent clinical success of *ex vivo* genetically modified CD34+ cells for X-SCID and ADA-SCID patients using retrovirus transduction has demonstrated the feasibility of the *ex vivo* approach (1, 2) and has brightened the gene therapy field. However, this approach has limitations, such as the size of recombinant DNA that can be packaged (3, 4), the potential of generating replication-competent viruses during vector production, recombination between the therapeutic virus and endogenous retroviral genomes, and the potential generation of infectious agents with novel cell specificity, host ranges, increased virulence and cytotoxicity (5, 6). Additionally, the preparation of fully qualified clinical-grade viral vector requires extensive resources and production time. A better approach to provide gene products, particularly one that avoids the risks associated with presently available methods and provides long-term production, would be valuable.

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Electroporation has been established as an efficient delivery method for loading a wide range of cell types (7-12). Numerous studies have shown that electroporation is capable of mediating transgene expression and cellular uptake of various molecules *in vitro* (13-18) *ex vivo* (19, 20, 21) and *in vivo* (22-26). Because it is a physical approach (7, 10, 27), electroporation-mediated *ex vivo* delivery of plasmid DNA avoids virus-related complications described above, accommodates large DNA size, and is time- and labor- efficient compared to systems employing viral vectors or chemicals/lipids. Electroporation is also potentially a less toxic method. For those cells that are very sensitive to electroporation, it was shown that the apparent toxicity was not related to electroporation itself, but to the uptake of DNA-mediated apoptosis (13, 28, 29). With these advantages in mind, traditional, commercially available static electroporation is widely used in biotechnology laboratories but is restricted to volumes typically less than 1 mL, which causes limitation when large volumes of cells need to be loaded. Recently, it was reported in a clinical trial that multiple cuvettes were used to electroporate activated human peripheral blood mononuclear cells (PBMCs) to modify human T lymphocytes (19). In that study, the transfected cells needed to be pooled and placed under drug selection for several months in order to generate high expression clones and to obtain adequate cell dosage for patients. A similar clinical trial employed electroporation of patients' fibroblasts with a plasmid encoding for Factor VIII-containing again followed by drug selection and screening for high expression clones (30). Most recently, it was reported that approximately 200 to 500 static cuvettes were necessary and the transfected cells were pooled in order to generate a library for functional genomic studies (31). Such multiple uses of cuvettes in static electroporation add not only labor and cost but also variability to the process. Though the above applications were achievable with static electroporation, an efficient, consistent, large volume transfection system is needed to satisfy industrial scaling-up for transient transfection, such as production of proteins and viral vectors, including retrovirus, lentiviral and adeno-associated virus.

To meet the needs for large volume transfection, we have focused on flow electroporation, an idea which originated in the mid 1980's (32). Flow electroporation (EP) permits cells to be electroporated while they flow between two electrodes and a transient electrical field is experienced by the cells as they pass between these electrodes. This method is convenient because it is based on automated (continuous or step-wise) replacement of the cell suspension between electrodes. Flow EP has been used to mediate transgene expression and molecule uptake by nucleated cells (33, 34), incorporation of inositol hexaphosphate (IHP) into red blood cells (35), and selection of hematopoietic cells and reduction of tumor cell contamination (20). For the two reports concerning flow

electrotransfection, the flow chambers had either a 4 mm (32) or 0.2 mm (36) electrode gap. Cells were electroporated with pulse widths in the millisecond range and electrical fields of a few hundreds of Volts per centimeter. Pulsing buffer with either low conductivity sucrose medium (32) or high conductivity phosphorylated serum medium (36) were used, respectively. However, to date, the transfection efficiencies reported (32,36) were no greater than 35% for adherent cells and less than 25% for suspension cells, which were about 50% of the values obtained with static electroporation (32). In this report, we describe efficient, large volume flow electroporation. The practical utility of flow electrotransfection in an *ex vivo* approach was supported by our demonstration that the transfected cells were viable and expressed the functional gene product *in vitro* and *in vivo* and demonstrated a biological effect.

Materials and Methods

Cell Lines

Suspension human acute T leukemia Jurkat (ATCC no. CRL-1990) and adherent mouse embryonic fibroblast 10T^{1/2}(ATCC no. CCI-226) cells were obtained from the American Type Culture Collection (Manassas, VA). Jurkat cells were cultured in complete medium consisting of RPMI-1640 supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (FBS, HyClone, Logan, UT). 10T^{1/2} cells were cultured in complete medium consisting of Dulbecco's MEM (DMEM) with the same supplement. The adherent human liver cell line, Huh-7, was obtained from Dr. Michael Blaese from PreGentis (Newtown, PA) and was maintained in DMEM supplemented with 1mM sodium pyruvate and the same supplement as described above. Cells were maintained in logarithmic growth phase at 37° C in a humidified atmosphere supplemented with 5% CO₂. All cell culture reagents were purchased from BioWhittaker (Walkersville, MD). All other chemical reagents were from Sigma (St. Louis, MO).

Plasmids

Plasmid pCI-eGFP was constructed by removal of eGFP cDNA from pEGFP-N1 plasmid (Clontech Laboratories, Palo Alto, CA) and subcloning the eGFP into pCI under the control of a cytomegalovirus (CMV) early promoter with an intron. pGEG.mIL12 and pGEG.eGFP were kind gifts from Dr. Osam Mazda and were described previously (37). All plasmids were propagated in *E. coli* strain DH5 α (Invitrogen) and purified on endotoxin-free Qiagen-tip 10000 columns (Qiagen, Chatsworth, CA). Each batch of plasmid DNA was routinely checked for its A260/A280 ratio, endotoxin level, and percentage of super coiled population, which typically are 1.75 to 1.9, 3 to 22 EU/mg, and 80 to 95% respectively.

Flow Electroporation

The MaxCyte GT™ system consisting of a power/switch module and a disposable flow pulsing chamber, were developed and assembled at MaxCyte, Inc. (Rockville, MD). A schematic drawing is shown in Figure 1. The pulsing chamber comprises two electrodes made of stainless steel with gold coating, separated by a non-conductive spacer made of silicon. The length of the electrode chamber was 3.5-5 cm, the width was 5 mm, and the parallel electrodes were 5 mm apart. The power/switch module (not shown) controlled by a computer can accommodate a wide range of electric pulse requirements, such as voltage (0 to 1000V), pulse shape (monophasic and biphasic square pulses) and width (10 μ s to 10 ms), interval between pulses (10 μ s to 10 s). The desired pulse number/cell P_N was calculated under assumption of piston-like motion of fluid and was achieved by applying pulses with certain pulse interval T . The T was determined by

$$T = Vol_{EP} / Q \cdot P_N$$

where Vol_{EP} is the volume of electroporation channel (milliliters) and Q stands for the flow rate (milliliters per second). In this report an average linear velocity of cells along the channel was about 0.5 cm/s.

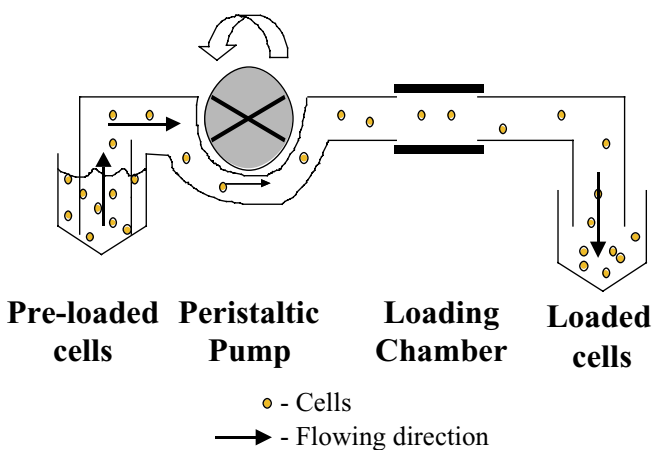


Figure 1: A schematic of the flow electroporation system. Target cells are first suspended in pulsing buffer together with target molecules. The mixture is pumped through the disposable flow chamber. Cells are electroporated while flowing in the chamber.

Immediately before electroporation, target cells were harvested either directly for suspended cells or after trypsinization for adherent cells and pelleted by centrifugation for 10 minutes at 250 x g at room temperature and washed once with pulsing buffer (125 mM KCl, 15 mM NaCl, 3 mM Glucose, 25 mM HEPES, 1.2 mM $MgCl_2$, pH 7.4). Cells were then resuspended in pulsing buffer at 1 to 8 x 10⁷ cells/mL together with either DNA plasmid at a concentration ranging from 30 to 80 μ g/mL, or 0.5 mg/mL of dextran (MW = 500KD) conjugated

with FITC. The cell/molecule mixture (volume 1.5 to 50 mL) was then pumped into the disposable flow chamber by peristaltic pump and electroporated during flowing. It is well known from practice that different cell types/lines have different sensitivity to electrical fields. After optimization, electrical fields were set at 1 kV to 2.3kV/cm, depending upon the cell line, to achieve highest efficiency with greater than 70% viability. The flow rate and pulsing sequence were synchronized such that each cell received 4 square pulses, 400 μ s each. The processed cells were collected and incubated at 37° C for 30 min before plating in complete medium.

Determination of Transfection Efficiency and Viability by Flow Cytometry

To evaluate eGFP transfection, cells were analyzed 40-48 hr post processing, unless otherwise specified. Briefly, transfected cells were washed in PBS, and incubated with propidium iodide at 1 μ g/mL immediately prior to flow cytometric analysis. Transfection efficiency was determined as the percentage of viable GFP-positive cells among total cells. Non-electroporated cells were gated as control with less than 0.5% of GFP positive cells. Viable cells were determined by propidium iodide (PI) exclusion test. Viability was defined as the percentage of PI-negative cells among total cells. For FITC-dextran uptake, the loaded cells were analyzed 3 hours post electroporation. The FACScan flow cytometer and CellQuest software were from Becton Dickinson Immunocytometry Systems (San Jose, CA).

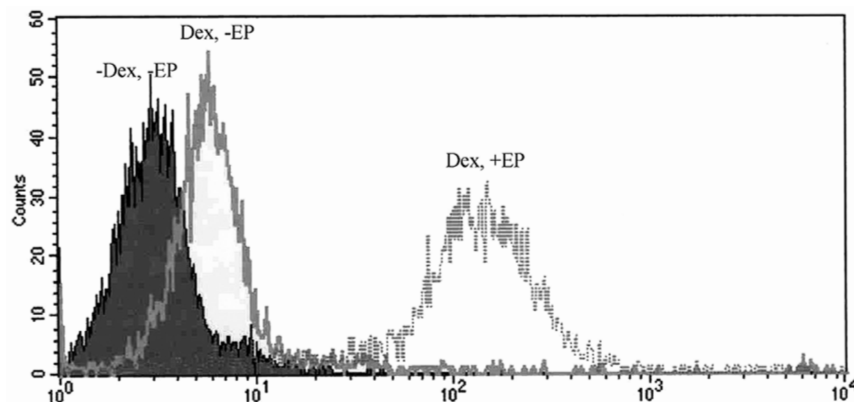
Angiogenesis Assay in Vivo

The effect of mIL12 transfected cells on new blood vessel formation was assessed in the MatriGel assay, performed as described previously (38). Briefly, male C3H mice (The Jackson Laboratory) were randomly divided in four groups (five animals/group): control, 1x10⁶ cells, 5x10⁶ cells, or 1x10⁶ cells in MatriGel. Animals received injections subcutaneously with 0.5 mL of MatriGel (BD Bioscience, Bedford, MA) supplemented with 1 μ g/mL bFGF (R&D Systems, Minneapolis, MN) on dorsal thorax area. Control or mIL12 transfected cells were subcutaneously injected at a remote site from the MatriGel injection (on ventral side close to thigh). In one group of mice, the transfected cells were mixed together with bFGF and MatriGel before injection. Blood was collected from each mouse 2 days after injection through retro-orbital puncture. Plasma was collected by centrifugation and stored at -80° C until use. Mouse systemic mIL12 level was analyzed using a commercially available ELISA kit (R&D System). Mice were sacrificed 7 days after the MatriGel injection. The gels were recovered by dissection and homogenized in 1 mL of PBS. The amount of hemoglobin in MatriGel was analyzed by a commercially available hemoglobin analysis kit (Sigma, St. Louis, MO), and served as an indicator of angiogenesis.

Results and Discussion

Flow Electroloading of Macromolecule

We first demonstrated that the flow electroporation could mediate sufficient electroloading of macromolecules into cells. We chose FITC conjugated dextran with a molecular weight of 500KD as a probe since its size is comparable to that of most antibodies or antigens. Approximately 2.5×10^8 Jurkat cells were resuspended in 5mL of electroporation buffer mixed with FITC-dextran (0.5 mg/mL). The loaded cells were analyzed by FACS and the results are shown in Figure 2. A right shift on the X-axis indicates increased uptake of FITC-dextran in electroporated cells (Dex, +EP). A slight right shift was observed on the cells incubated with FITC-dextran (+Dex, -EP) which reflected a non-specific binding of the molecule. The electroporated cells had an increased mean fluorescence intensity (Table I). Flow electroporation can thus mediate an efficient uptake of macromolecules with greater than 90% of efficiency, and 90% of cell viability (Table I). The successful flow electroloading of FITC-dextran (500kD) indicated that flow electroloading of other macromolecules, such as antibodies and antigens, into cells would be feasible.



Flow Electrotransfection

We next examined the ability of flow electroporation to transfect mammalian cells for gene expression. A plasmid carrying the gene coding for enhanced green fluorescence protein (eGFP), driven by the CMV promoter, was used. As shown in Figure 3, various volumes of Jurkat cells ranging from 1.5 to 50 mL were flow electrotransfected by 4 pulses at 1 kV/cm with 400 microseconds pulse width. Samples of Jurkat cells with various volumes (1.5, 5, 10, 15, 50 mL), when processed in the same flow channel with the same flow rate and pulsing settings, had comparable transfection efficiencies, ranging between 70 to 76%, and no significant differences in cell viability, as measured by PI exclusion (80 to 90%). In this experiment, we clearly showed the advantage of flow electrotransfection in that it can mediate effi-

Table I

Flow Electroporation-mediated Uptake of FITC-Dex(500kD)

Jurkat cells were flow electroporated as described in Figure 1. Flow cytometric analysis of FITC-dextran for FITC-dextran loading efficiency and propidium iodide exclusion for cell viability was performed 3h after electroporation. Data is presented as mean and standard deviation of the duplicated experiments.

	- EP, - Dex	- EP, Dex	EP + Dex
Viability (%)	95	92±2	92±1.4
Fluorescent cells (%)	0.1	35±20	90±1.4
Mean Fluorescence Intensity	6	76±53	185±22

cient transfection of a large volume of cells (up to 50 mL) with high cell viability, and that it has great potential in industrial bioprocesses.

One of the goals of this study was to test durability of the electrodes. Unlike the electrodes in a disposable electroporation cuvette, the electrodes used in a flow channel must carry a bigger load because of repetitive pulsing during the process. For example, it took 200 discharge cycles to electroporate 50 mL of cell suspension. Electrical current flowing through an electrode surface during each voltage pulse

Figure 2: Flow cytometric analysis of flow electroloading of FITC-Dextran. 5mL of Jurkat cells (approximately 2.5×10^8) was flow electroporated with four 1.2 kV/cm, 400 μ s pulses/cell (2.25s pulse interval at 0.1mL/s of flow rate) in the presence of 0.5mg/mL of FITC-Dextran (MW=500KD). Three hours post electroporation, flow cytometric analysis of FITC-dextran was performed to estimate cell-loading efficiency of dextran electroporated cells (Dex, +EP), dextran incubated cells (Dex, -EP) and non-dextran cells (-Dex, -EP).

is associated with electrochemical reactions taking place at the electrode surface. Products of these reactions can be detrimental to cells and affect their viability and transfection efficiency. However, Figure 4 shows that there was no significant difference in efficiency and cell viability among samples collected at different time points during the process. This suggests that long time pulsing and repeatable usage of the electrodes did not result in production of harmful chemical byproducts in the cell suspension. The flow electroporation is able to mediate reliable and consistent transfection and it is equipped for scaling up. For the 50 mL experiment, the flow electrotransfection processing took about 9 min. This is the first demonstration of flow electrotransfection of 50 mL of cell suspension, and most importantly, high transfection efficiency and cell viability were achieved in the process.

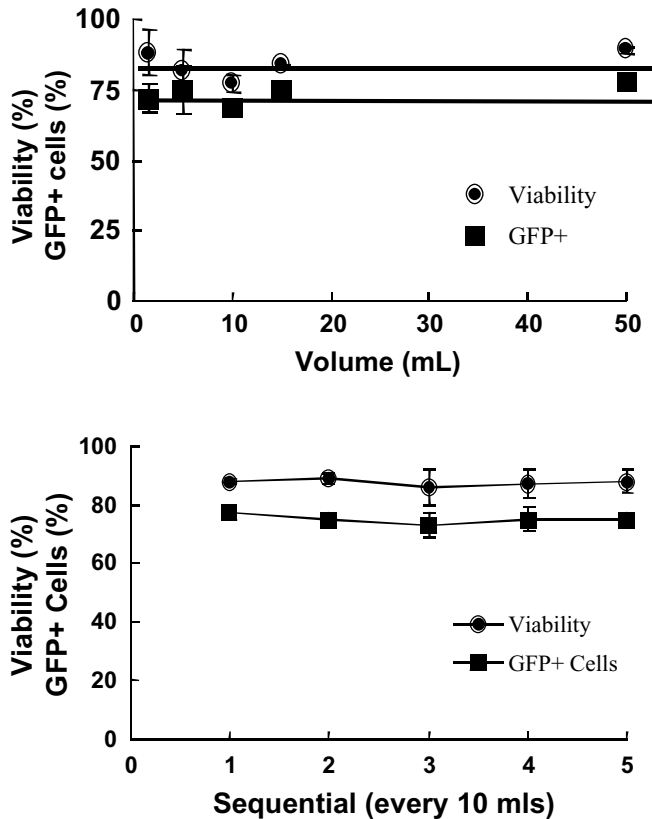


Figure 3: Flow electrotransfection of large volume of Jurkat cells. Jurkat cells at the volume of 1.5, 5, 10, 15, 50 mL, respectively, were flow electrotransfected with 4 pulses/cell at 1 kV/cm, 400 μ s pulse width (2.3s pulse interval at 0.1mL/s of flow rate) in the presence of DNA plasmid encoding eGFP reporter gene (80 μ g/mL) driven by a CMV promoter. Flow cytometric analysis of eGFP for transfection efficiency and propidium iodide exclusion for cell viability was performed 48h after electrotransfection. Error bars denote mean and standard deviation of data from more than 3 repeats, except for samples of 50 ml cells, where error bar denote mean and standard deviation of data from duplicated experiments.

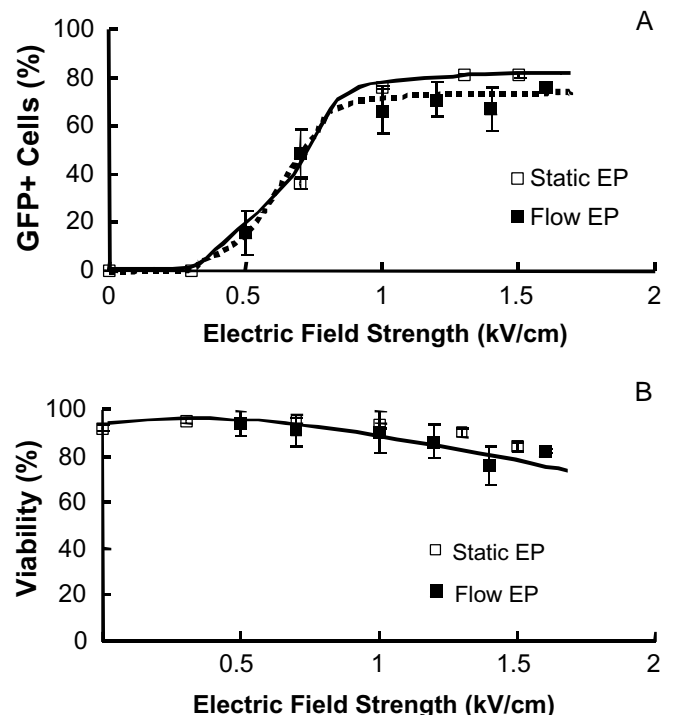
Figure 4: Consistence of flow electrotransfection. 50 mL of Jurkat cells (2.5×10^9 total cells) was flow electrotransfected with four 1 kV/cm, 400 μ s pulses/cell during flowing (0.1 mL/s flow rate, 2.3 s as pulse interval) in the presence of 80 μ g/mL of DNA plasmid encoding eGFP reporter gene. The transfected samples were collected every 10 mL, and cultured in complete medium. Flow cytometric analysis of eGFP for transfection efficiency and propidium iodide exclusion for cell viability was performed 40 hours post electrotransfection. Error bars denote the mean and standard deviation from data of duplicated experiments.

Comparison of Flow and Static Electrotransfection

It was reported previously that efficiency of transfecting cells with a plasmid in a flow mode was approximately half that of static electrotransfection (32). We directly compared the outcome of static and flow electrotransfection by processing Jurkat cells with the eGFP DNA plasmid either in static or flow modes. Various electric field strengths, ranging from 0.4 to 1.7 kV/cm, were used. The pulsing conditions were identical in both static and flow modes. As shown in Figure 5, the transfection efficiency increased as the electric field strength was raised. There was a modest decrease in cell viability at the highest field strength of 1.7 kV/cm. However, there was no significant difference in either transfection efficiency or viability between the static and flow electrotransfection. Flow electrotransfection allows large volumes of cells to be transfected at high efficiency.

Figure 5: Comparison of electrotransfection efficiency and cell viability between flow and static electrotransfection. Jurkat cells were electrotransfected with four pulses, at 400 μ s pulse width in the presence of plasmid DNA encoding eGFP either in a static or flow (0.1 mL/s flow rate, 2.3 s as pulse interval) modes. Various electric field strengths ranging from 0.4 to 1.7 kV/cm were applied to the cells. Flow cytometric analysis of eGFP for transfection efficiency (Figure 5A) and propidium iodide exclusion for cell viability (Figure 5B) was performed 48 h after electrotransfection. Error bars denote mean and standard deviation of data from more than three experiments.

The fact that flow electrotransfection efficiency was comparable with that of static electrotransfection (Figure 5) indicated that under described conditions the velocity profile was rather uniform inside the flow chamber. Therefore we could use the flow rate and the volume of electrotransfection



area V_{EP} to derive the pulsing interval in order to apply the desired number of pulses to the flowing cells, as described in the Materials and Methods.

Large Volume Flow Electrotransfection of Both Suspended and Adherent Cells

After demonstrating that flow electroporation was able to mediate efficient transfection for suspended cells, we tested whether flow electrotransfection is also applicable to adherent cells. Two adherent cell lines, 10T1/2 and Huh-7 cells, were selected for flow electrotransfection with the GFP plasmids (Table II). Electric field strengths of 1.2 kV/cm and 1.6 kV/cm were applied to Huh-7 and 10T1/2 cells, respectively. The remaining parameters were identical to that of Jurkat described previously. Flow electrotransfection of adherent cells resulted in greater than 90% cell viability and greater than 63% transfection efficiency (Table II), which is considerably more efficient than previous reports. These results demonstrated that the flow electrotransfection system tested is as flexible as static systems and is applicable to most cell types.

Dependence of Flow Electroporation-mediated Transgene Expression on DNA Plasmid

For gene therapy, there is a wide spectrum of requirement for duration of gene expression. It is known that electrotransfection, in the absence of selection, typically results in transient expression of the transgene. Such transient expression may limit the potential of electroporation for some gene therapy applications. To improve the duration of transgene expression mediated by electrotransfection, we tested a plasmid construct carrying the EBNA1-oriP region from EBV, which was reported to prolong transgene expression (37). We compared the results from a standard plasmid DNA carrying eGFP reg-

ulated by the CMV promoter to those from an EBNA1 containing plasmid carrying eGFP regulated by the CAG promoter (pGEG.eGFP). The morphology of the transfected Jurkat cells 4 days post flow electrotransfection with pGEG.eGFP is shown in Figure 6A. The high cell viability and transfection efficiency of flow electrotransfected cells shown in Figure 6A were confirmed by FACS analysis (Figure 6B). Standard DNA plasmid, pCMV-eGFP, caused a very transient GFP expression. The expression level decreased dramatically by 2

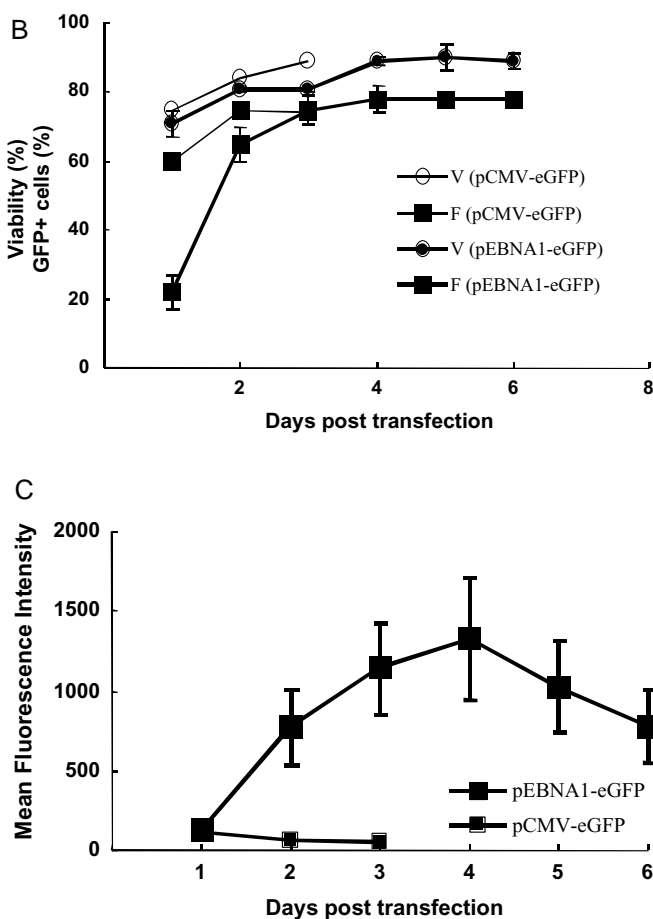
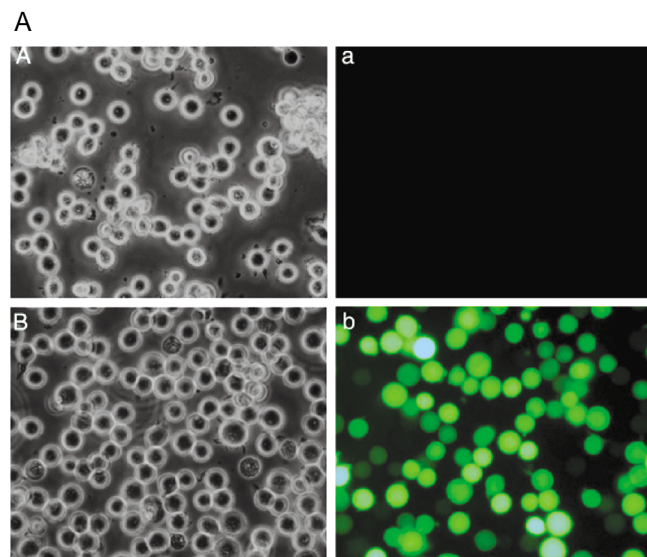


Figure 6: Plasmid effect on flow electrotransfection. Jurkat cells were electrotransfected with four 1 kV/cm 400 μ s pulses/cell during flowing (0.1 mL/s flow rate, 2.3 s as pulse interval) in the presence of either standard plasmid DNA (pCMV-eGFP, 80 μ g/mL), or plasmid DNA containing EBNA1-OriP region (pGEG-eGFP, 100 μ g/mL) from EBV virus. The transfected cells were analyzed by microscope and flow cytometry for morphology, transfection efficiency, viability and transgene expression level (mean fluorescence intensity). Figure 6A showed the morphology of the transfected cells. A and B are the phase contrast micrographs. a and b are the fluorescence micrographs of the corresponding fields of A and B, respectively. A and a are the control cells without electroporation and B and b are the cells with flow electrotransfection. Figure 6B revealed transfection efficiency and viability. Figure 6C demonstrated mean fluorescence intensity. Error bars denote mean and standard deviation of data from triplicated experiments.

days post electroporation, which may be due to the plasmid DNA being diluted or degraded during cell mitosis. However, the EBNA1 containing plasmid, pGEG.eGFP, gave rise to a longer term transgene expression (Figure 6C). The viability of pGEG.eGFP electroporated cells was comparable to that of the pCMV-eGFP transfection cells (Figure 6B). Although the transfection efficiency with pGEG.eGFP was much lower than the one with pCMV-eGFP at day 1 (Figure 6B), presumably due to the fact that pGEG.eGFP is a larger sized plasmid, the transfection efficiency quickly reached the similar levels and the expression level (mean fluorescence intensity) of pEBNA-1eGFP increased to a much higher level 2 days post EP (Figure 6C). The GFP expression level of pGEG.eGFP was 500 to 1000 fold higher than the one of pCMV-eGFP (Figure 6C). These results demonstrate that, when long term expression is needed, properly modified plasmids may lessen or even overcome the transient expression commonly associated with electrotransfection. Moreover, recent studies have reported that with new recombinant DNA technologies, such as the DNA transposon Sleeping Beauty (40-43) and co-expression of a Phage integrase (44, 45), could increase transgene integration into host genome. The combination of our system with such new DNA vector systems has the potential to bring non-viral, long-term gene expression to reality. At present, flow electroporation-mediated transient gene expression can be applied in cancer immunotherapy and angiogenesis therapy where long term expression is not desired.

In vivo Efficacy of Flow Electroporated Cells

To demonstrate the feasibility of using flow electrotransfected cells, in large volumes, as an *in vivo* gene delivery vehicle, we evaluated the efficacy of mIL12 transfected cells in an angiogenesis model of Matrigel. In this model, recombinant bFGF is mixed in Matrigel, which is then injected to mouse subcutaneously. Matrigel becomes solidified and the bFGF attracts mouse endothelial cells, which forms micro blood vessel inside the Matrigel (38). Thus, angiogenesis can be analyzed by measuring hemoglobin concentration in the Matrigel (38). Mouse IL-12 has been shown to up-regulate IFN γ and IP10, and to block angiogenesis (46). We believe that Matrigel angiogenesis model is an appropriate model for testing whether transfected cells with mIL-12 gene express functional mIL-12 *in vivo*. C3H mouse embryonic fibroblast 10T1/2 cells were flow electrotransfected with pGEG.mIL12 and injected into mice subcutaneously, as described in Materials and Methods. *In vitro*, the transfected cells secreted approximately 400 ng of mIL-12, as determined by ELISA (1×10^6 cells in 24 hours). *In vivo*, mouse systemic mIL-12

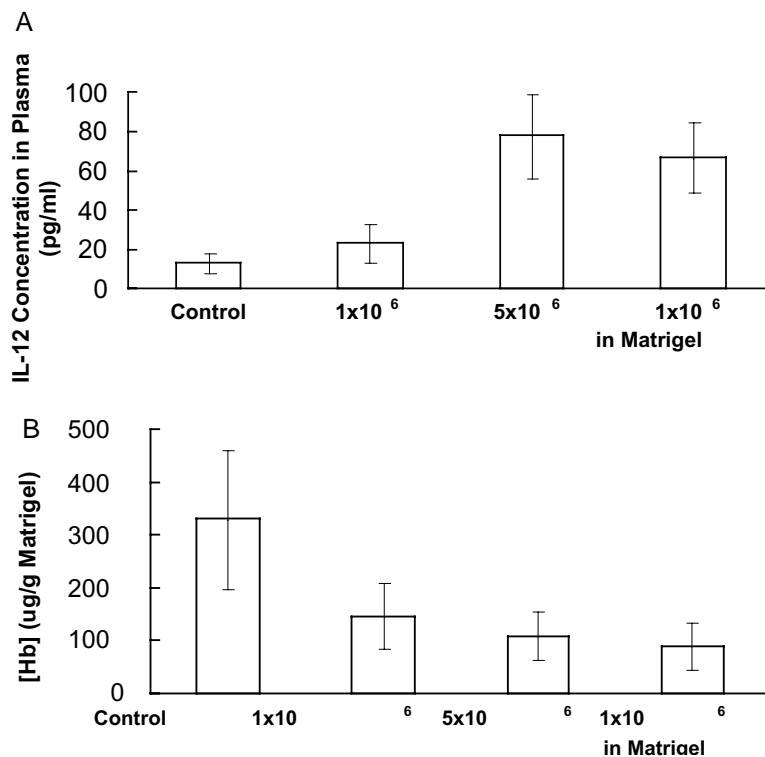


Figure 7: mIL-12 transfected mouse embryonic fibroblast blocked bFGF induced angiogenesis *in vivo*. Three hrs post flow electrotransfection with two 2.3 kV/cm 800 μ s pulses/cell in the presence of plasmid DNA containing EBNA1-oriP region encoding mIL12, mouse embryonic 10T1/2 cells (1×10^6 or 5×10^6) were subcutaneously injected into C3H mice on the ventral side close to the thigh, except for cells injected in the mixture with Matrigel. Matrigel containing 1 μ g/mL bFGF was subcutaneously injected into the dorsal thorax area as described in the Materials and Methods. Control represents mice injected with non-transfected cells. Figure 7A is result of ELISA analysis (R&D Systems) of mIL-12 in plasma 2 days post injection. Figure 7B shows the accumulated hemoglobin in Matrigel plugs 7 days post injection. Error bars denote mean and standard deviation of data from 5 mice.

level was significantly elevated for mice receiving injection of 5×10^6 mIL-12 transfected cells or 1×10^6 mIL-12 transfected cells in Matrigel (Figure 7A, $p < 0.05$, Students t test). Furthermore, the hemoglobin concentration in Matrigel significantly decreased among all mice receiving mIL-12 transfected cells (Figure 7B, $p < 0.05$, Students t test). The systemic mIL12 level correlated with the degree of inhibition of angiogenesis. As shown in Figure 7B, mIL-12 transfected cells inhibited angiogenesis greater than 70% with either a 5 million cell dosage, or a 1 million cell dosage locally administered (inside the Matrigel). This is the first demonstration that flow electrotransfected cells are viable and express the functional gene product *in vitro* and *in vivo*, validating the application of this system for gene therapy.

Conclusions

We have shown that high transfection efficiency was achieved

by flow electroporation using cell volumes up to 50 mL, with cell concentrations ranging from 1 to 8×10^7 cells/mL. The efficiency by flow electrotransfection was similar to that obtained by a static process. Up to 50 mL with cell number up to 2.5×10^9 can be transfected in less than 10 minutes and the system is flexible for scaling-up. The flow electrotransfected cells were functional for a sustained period post *in vivo* transplantation and produced functional gene products. We believe this non-viral gene transfer platform can play an important role in clinical gene based therapies. The large volume, high speed and high efficiency should also benefit industrial bioprocesses.

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References and Footnotes

- Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., Fischer, A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 28, 669-72 (2000)
- Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M. G., Bordignon, C. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 28, 2410-3 (2002)
- Miller, A. D. Retrovirus packaging cells. *Hum. Gene Ther.* 1, 5-14 (1990)
- Miller, A. D. Retrovirus vectors. *Curr. Top. Microbiol. Immunol.* 158, 1-24 (1992)
- Dyer, M. R., Herrling, P. L. Progress and potential for gene-based medicines. *Mol Ther* 1, 213-24 (2000)
- Daly, G., Chernajovsky, Y. Recent developments in retroviral-mediated gene transduction. *Mol Ther* 2, 423-34 (2000)
- Hui, S.W. and Li, L.H. *In vitro* and *ex vivo* delivery of genes to cells by electroporation. In: *Electrically mediated delivery of molecules to cells: electrochemotherapy, electrogene therapy and transdermal delivery by electroporation*. (Jaroszeski M.J., Gilbert R. And Heller R. Eds. Humana Press, Totowa, NJ). p157-172 (2000)
- Sabri, N., Pelissier, B. and Teissie, J. Electroporation of intact maize cells induces an oxidative stress. *European J. Biochem.* 238, 737-743 (1996)
- Salek, A., Schnettler, R. and Zimmermann, U. Stably inherited killer activity in industrial yeast strains obtained by electrotransformation. *FEMS Microbiology Letters* 75, 103-109 (1992)
- Tsong, T. Y. Electroporation of cell membranes. *Biophys. J.*, 60, 297-306 (1991)
- Xie, T. D., Sun, L. and Tsong, T. Y. Study of mechanisms of electric field-induced DNA transfection I. DNA entry by surface binding and diffusion through membrane pores. *Biophys. J.* 58, 13-19 (1990)
- Neumann, E., Schaefer-Ridder, M., Wand, Y. and Hofschneider, P. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *The EMBO Journal* 1, 841-845 (1982)
- Li, L. H., Sen, A., Murphy, S. P., Jahreis, G. P., Fuji, H. and Hui, S. W. Apoptosis induced by DNA uptake limits transfection efficiency. *Experimental Cell Research* 253, 541-550 (1999)
- Prasnitz, M. R., Milano, C. D., Gimm, J. A., Langer, R. and Weaver, J. C. Quantitative study of molecular transport due to electroporation: uptake of bovine serum albumin by erythrocyte ghosts. *Biophys. J.* 66, 1522-1530 (1994)
- Klenchin, V. A., Sukharev, S. I., Serov, S. M., Chernomordik, L. V. and Chizmadzhev, Yu. A. Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys. J.* 60, 804-811 (1991)
- Chang, D. C., Guo, P. Q. and Maxwell, B. L. High efficient gene transfection by electroporation using a radio-frequency electric field. *Biochim. Biophys. Acta* 1992, 153-160 (1991)
- Liang, H., Purucker, W. J., Stenger, D. A., Kubinieć, R. T. and Hui, S. W. Uptake of fluorescence-labeled dextrans by 10T1/2 fibroblasts following permeation by rectangular and exponential-decay electric field pulses. *BioTechniques* 6, 550-558 (1988)
- Stopper, H., Jones, H. and Zimmermann, U. Large scale transfection of mouse L-cells by electroporation. *Biochim. et Biophys.* 900, 38-44 (1987)
- Jensen, M. C., Clarke, P., Tan, G., Wright, C., Chung-Chang, W., Clark, T. N., Zhang, F., Slovak, M. L., Wu, A. M., Forman, S. J., Raubitschek, A. Human T lymphocyte genetic modification with naked DNA. *Mol. Ther.* 1, 49-55 (2000)
- Eppich, H. M., Foxall, R., Gaynor, K., Dombkowski, D., Miura, N., Cheng, T., Silva-Arrieta, S., Evans, R. H., Mangano, J. A., Preffer, F. I., Scadden, D. T. Pulsed electric fields for selection of hematopoietic cells and depletion of tumor cell contaminants. *Nat. Biotechnol.* 18, 882-7 (2000)
- Bergan, R., Hakim, F., Schwartz, G. N., Kyle, E., Cepeda, R., Szabo, J. M., Fowler, D., Gress, R. & Neckers, L. Electroporation of synthetic oligodeoxynucleotides: a novel technique for *ex vivo* bone marrow purging. *Blood* 88, 731-741 (1996)
- Heller, L. C., Ingram, S. F., Lucas, M. L., Gilbert, R. A., Heller, R. *Technology in Cancer Research. & Treatment* 1, 205-209 (2002)
- Wells, J. M., Li, L. H., Sen, A., Jahreis, G. P., Hui, S. W. Electroporation-enhanced gene delivery in mammary tumors. *Gene Ther.* 7, 541-547 (2000)
- Rols, M. P., Delteil, C., Golzio, M., Dumond, P., Cros, S. and Teissie, J. *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nature Biotechnology* 16, 168-71 (1998)
- Hofmann, G. A., Dev, S. B., Nanda, G. S., Rabussay, D. Electroporation therapy of solid tumors. *Crit. Rev. Ther. Drug. Carrier. Syst.* 16, 523-69 (1999)
- Heller, R. Treatment of cutaneous nodules using electrochemotherapy. *J. Fla. Med. Assoc.* 82, 147-50. Review. (1995)
- Neumann, E., Toensing, E., Kakorin, S., Budde, P. and Frey, J. Mechanism of electroporative dye uptake by mouse B cells. *Biophys. J.* 74, 98-108 (1998)
- Li, L. H., McCarthy, P., and Hui, S. W. High-efficiency electrotransfection of human primary hematopoietic stem cells *FASEB J*, 15, 586-8 (2001)
- Shimokawa, T., Okumura, K., Ra, C. DNA induces apoptosis in electroporated human promonocytic cell line U937. *Biochem. Biophys. Res. Commun.* 270, 94-9 (2000)
- Roth, D. A., Tawa, N. E., Jr, O'Brien, J. M., Treco, D. A., Selden, R. F. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N. Engl. J. Med.* 344, 1735-42 (2001)
- Harrington, J. J., Sherf, B., Rundlett, S., Jackson, P. D., Perry, R., Cain, S., Leventhal, C., Thornton, M., Ramachandran, R., Whittington, J., Lerner, L., Costanzo, D., McElligott, K., Boozer, S., Mays, R., Smith, E., Veloso, N., Klika, A., Hess, J., Cothren, K., Lo, K., Offenbacher, J., Danzig, J., Ducar, M. Creation of genome-wide protein expression libraries using random activation of gene expression. *Nat. Biotechnol.* 19, 440-5 (2001)

32. Rols, M. P., Coulet, D., Teissie, J. Highly efficient transfection of mammalian cells by electric field pulses. Application to large volumes of cell culture by using a flow system. *Eur. J. Biochem.* 206, 115-21 (1992)
33. Li, L. H., Wang, S., Wood, M., Singh, V., Fratantoni, J., and Liu, L. N. Non-viral gene transfer into resting primary lymphocytes: small and large volume. *Mol. Ther.* 3, S63 (2001)
34. Li, L. H., Feller, S., Shivakumar, R., Wood, M., Wang, S., Singh, V., Holaday, J., Fratantoni, J., Liu, L. N. High Flow Electroporation: a Novel Approach For *Ex Vivo* Gene Therapy. *Blood* 98, 425a (2001)
35. Bruggemann, U., Roux, E. C., Hannig, J., Nicolau, C. Low-oxygen-affinity red cells produced in a large-volume, continuous-flow electroporation system. *Transfusion* 35, 478-86 (1995)
36. Lin, Y.-C., Jen, C.-M., Huang, M.-Y., Lin, X.-Z. Flow-type electroporation chips for gene transfection. *Proceedings of SPIE.* 4177, 194-198 (2000)
37. Cui, F. D., Kishida, T., Ohashi, S., Asada, H., Yasutomi, K., Satoh, E., Kubo, T., Fushiki, S., Imanishi, J., Mazda, O. Highly efficient gene transfer into murine liver achieved by intravenous administration of naked Epstein-Barr virus (EBV)-based plasmid vectors. *Gene Ther.* 8, 1508-13 (2001)
38. Jin, X., Bookstein, R., Wills, K., Avanzini, J., Tsai, V., LaFace, D., Terracina, G., Shi, B., Nielsen, L. L. Evaluation of endostatin antiangiogenesis gene therapy *in vitro* and *in vivo*. *Cancer Gene Ther.* 8, 982-9 (2001)
39. Zimmermann, U. Electrical breakdown, electroporation and electrofusion. *Rev. Physiol. Biochem. Pharmacol.* 105, 175-256 (1986)
40. Kaminski, J. M., Huber, M. R., Summers, J. B., Ward, M. B. Design of a nonviral vector for site-selective, efficient integration into the human genome. *FASEB J* 16, 1242-7 (2002)
41. Richardson, P. D., Augustin, L. B., Kren, B. T., Steer, C. J. Gene repair and transposon-mediated gene therapy. *Stem Cells* 20, 105-18 (2002)
42. Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., Kay, M. A. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat. Genet.* 25, 35-41 (2000)
43. Cui, Z., Geurts, A. M., Liu, G., Kaufman, C. D., Hackett, P. B. Structure-function analysis of the inverted terminal repeats of the sleeping beauty transposon. *J. Mol. Biol.* 17, 1221-35 (2002)
44. Groth, A. C., Olivares, E. C., Thyagarajan, B., Calos, M. P. A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* 97, 5995-6000 (2000)
45. Thyagarajan, B., Olivares, E. C., Hollis, R. P., Ginsburg, D. S., Calos, M. P. Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol. Cell. Biol.* 21, 3926-34. (2001)
46. Ando, D. Gene therapy and molecular immunology unite to give new life to cancer immunotherapy. *Mol. Ther.* 5, 665-7 (2002)
47. Factor, P. Gene therapy for acute diseases. *Mol. Ther.* 4, 515-24 (2001)
48. Pertl, U., Luster, A. D., Varki, N. M., Homann, D., Gaedicke, G., Reisfeld, R. A., Lode, H. N. IFN-gamma-inducible protein-10 is essential for the generation of a protective tumor-specific CD8 T cell response induced by single-chain IL-12 gene therapy. *J. Immunol.* 166, 6944-51 (2001)

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