TITLE:

Permeabilization of Adhered Cells Using an Inert Gas Jet

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SHORT ABSTRACT:

This protocol describes a method for the temporary permeabilization of adherent cells using an inert gas jet. This technique facilitates the transfer of genetic material and biomolecules into adherent mammalian cells by the utilization of mechanical forces to disrupt the plasma membrane.

LONG ABSTRACT:

Various cell transfection techniques exist and these can be broken down to three broad categories: viral, chemical and mechanical. This protocol describes a mechanical method to temporally permeabilize adherent cells using an inert gas jet which can facilitate the transfer of normally non-permeable macromolecules into adherent mammalian cells. We believe this technique works by imparting shear forces on the plasma membrane of adherent cells, resulting in the temporary formation of micro-pores. Once these pores are created, the cells are then permeable to genetic material and other biomolecules. The mechanical forces involved do run the risk of permanently damaging or detaching cells from their substrate. There is, therefore, a narrow range of inert gas dynamics where the technique is effective. An inert gas jet has proven efficient at permeabilizing various adherent cell lines including HeLa, HEK293 and human abdominal aortic endothelial cells. This protocol is appropriate for the permeabilization of adherent cells both *in vitro* and we have demonstrated its applicability *in vivo*, showing it may be used

for research and potentially in future clinical applications. It also has the advantage of permeabilizing cells in a spatially restrictive manner which could prove to be a valuable research tool. **INTRODUCTION:**

With the evolution of biomedicine and the understanding of cell mechanics, the delivery of biomolecules into cells has become vital to many research fields and medical therapies. Different techniques have been developed to introduce foreign molecules through the plasma membrane to the cell cytosol and these can be generally classified as either: viral, chemical or mechanical techniques. Viral techniques can transfect genetic material such as RNA and DNA using viral vectors[1]. Viral techniques tend to have high efficiencies in certain cell lines however they do have drawbacks. For example, Yang et al observed that undeleted viral genes from the adenovirus used for transduction were copied by the host hepatic cells leading to increased apoptosis[2]. Common chemical transfection methods include precipitation with calcium phosphate[3], bacterial exotoxins[4] and lipofection[5]. These techniques have been proven to be effective; however, certain problems arise such as cell toxicity and non-specificity. Furthermore, techniques such as calcium phosphate precipitation have been found to have transfection efficiencies up to 70% but only in certain cell lines, rarely in primary cells[6]. This is of note as increasing research efforts are being put into primary cells, especially in the case of designing various treatments for clinical use and the study of DNA functioning.

Temporal disruption of the cell membrane through mechanical stimuli is another method of introduction of foreign molecules into cells. Techniques include: microinjection of molecules[7], electroporation using an electric field to disrupt the membrane[8,9], sonoporation using ultrasound waves to disrupt the cell membrane[10-12], particle bombardment as demonstrated by the "gene gun" which shoots particles bound with genes into the cell[13] and more recently, the application of fluid shear stress has been shown to temporally permeabilze mammalian cells[14]. Although mechanical methods avoid some of the aforementioned issues, they are typically accompanied by lower efficiencies and very complex and specialized setups.

An atmospheric pressure glow discharge torch (APGD-t) was developed at McGill with the initial goal of functionalizing surfaces and detaching adherent cells *in vitro*[15]. Serendipitously, it was discovered that a live/dead stain being used was somehow being taken in by cells without a permeabilizing agent being added. Furthermore, this seemed to have occurred only in restricted areas of the wells which happened to match up with the torch path. Investigation into the permeabilization capabilities of the APGD-t continued and in control studies, it was found that the carrier inert gas jet of the plasma without excitation was also able to permeabilize cells. This contradicted the initial hypothesis that the reactive species created by the plasma jet temporarily impaired the membrane function and suggested that simply the mechanical forces were the cause of permeabilization. From here, studies continued in our lab to try and quantify and characterize the permeabilization efficiency of an inert gas jet as well as look at its transfection capabilities[15].

Through these studies, it has been found that micro-pores do in fact form in the plasma membrane, and these pores tend to reseal within approximately 5 seconds. We have demonstrated the technique's utility *in vitro* and *in vivo* in the chorioallantoic membrane of a chick embryo.

PROCEDURE:

1. Development of LabView® Program
   1.1. A mass flow controller (MKS M100B Mass-Flo Controller) is attached to the helium gas line to ensure a precise gas flow rate. This unit is controlled by an input voltage, ranging between 0 and 5 V. A control loop in the LabView® program determines the required voltage at any given time. Interfacing between the computer and the mass flow controller is performed by a data acquisition device (NI USB-6009) and a 12V power supply provides power to the mass flow controller.
   1.2. Two positioning linear slides are used to control the movement of the 6-well plate, one for each direction. Each assembly consists of a MA15-series Velmex® Unislide Assembly coupled with a stepping motor, and both assemblies are controlled by a single Velmex® VXM Stepping Motor Controller. The controller allows for manual jogging of each motor or accepts a string of external serial commands, the combination of which allow for specific movement patterns. Several patterns can be programmed into the LabView® program, requiring the user to provide only the desired torch speed as well as the path dimensions.
2. Preparation of Equipment
   2.1. Culture adherent cell line to confluence on glass cover slips in a 6-well plate using standard culturing techniques. For HeLa cells, seed 6-well plates with $2x10^5$ cells per well and incubate for 48 hours before treatment.
   2.2. Prepare appropriate volume of solution containing the desired vector. For hrGFPII-1, a concentration of 50ng/µL in 1X PBS provides a strong fluorescent signal in HeLa cells 24 hours after transfection. For dextran studies, 80µg/mL of 10kDa green fluorescent dextran is recommended. Hence forth, this solution will be referred to as "permeabilizing solution".
   2.3. Open both the helium cylinder and regulator.
   2.4. Turn on the motor controller.
   2.5. Open the LabView® program and verify all conditions. Note: Inputting too large of a well diameter will cause the capillary nozzle to break.
   2.6. Run the program. You will be asked if the platform is centered. To check, look at each motor stage and verify that each carriage is not near either stage end. Either let the program center the carriages or manually adjust by using the jogging controls on the front of the motor controller.
   2.7. If this is the first run of the day, the mass flow controller requires a warm-up. You may either run the program once without cells, or you may select “Run MFC” followed by “Stop MFC” after a minute or so. Note: Be sure to use a non-zero flow rate.
3. Preparation of the Gas Jet Capillary Nozzle Height
   3.1. Remove the capillary from the holder and zero the height dial.
   3.2. Place the 6-well plate on the platform.
   3.3. Replace and lower the gas jet capillary to the bottom of the well until it hits the cover slip. Secure the capillary in the holder.
   3.4. Using the height dial, raise the capillary 3mm. Mark this height on the capillary base.
   3.5. Raise the capillary to a safe height and then remove the 6-well plate.

4. Cell Permeabilization Protocol
    4.1. Remove cell culture media from well and rinse three times with 1X PBS.
    4.2. Pipette 1370µL of permeabilizing solution prepared in step 2 into well. This should result in a liquid depth of approximately 1.3mm.
    4.3. Place the well containing the permeabilizing solution in the center of the platform. Lower the gas jet nozzle to the previously marked level indicating a nozzle height of 3mm above the slide.
    4.4. Set helium flowrate in the LabView® program and select the desired movement pattern to be used. Select "Run" when ready to begin the permeabilization protocol. There will be an initial lag period during which the mass flow controller is preparing to provide the necessary flowrate. Once the appropriate flow rate has been reached, then the LabView® program will activate the stepper motors to move the well in the desired permeabilization pattern. Once the platform has stopped, wait approximately 30 seconds and remove the permeabilizing solution. Note: Optimal permeabilization occurs near a dynamic pressure of 100 to 200Pa at the nozzle outlet, depending on the capillary diameter.
    4.5. Wash well three times with 1X PBS.
    4.6. Fill well with fresh culture media.
    4.7. Repeat steps 4.1-4.6 for desired number of experiments. Note: Be sure to include control samples which consist of wells being filled with the permeabilizing solution without the gas jet being run over them. Leave the solution in the control wells for approximately 1 minute then proceed to steps 4.5 and 4.6.
    4.8. If running an hrGFP transfection experiment, return 6-well plate to incubator for 24 hours to allow transfected material to be transcribed by the cells. If running a dextran permeabilization experiment, return 6-well plate to incubator for 15 minutes.
5. Cell Imaging
    5.1. If desired, immediately before imaging, counterstain with appropriate live/dead stain to evaluate cell death.
    5.2. Image cells with appropriate microscope to investigate transfection/ permeabilization efficacy.

REPRESENTATIVE RESULTS:

Permeabilzation of HeLa cells with 10kDa green fluorescent dextran using a helium gas jet with a 0.86mm inner diameter capillary is shown in Figure 1. Cells were counterstained immediately after permeabilization with 2µL/mL EthD-1 solution (LIVE/DEAD® Viability/ Cytotoxicity Kit) to visualize cell death. Immediately following counterstaining, cover slips were mounted and imaged. This figure shows results of running the helium gas jet at three different outlet pressures compared to a control sample. Note that the permeabilization track width and efficiency increased with a higher dynamic pressure and cell death only showed a slight increase (A and B). However, once a high dynamic pressure was reached, HeLa cells were stripped from the cover slips and very little peripheral permeabilization occurred.

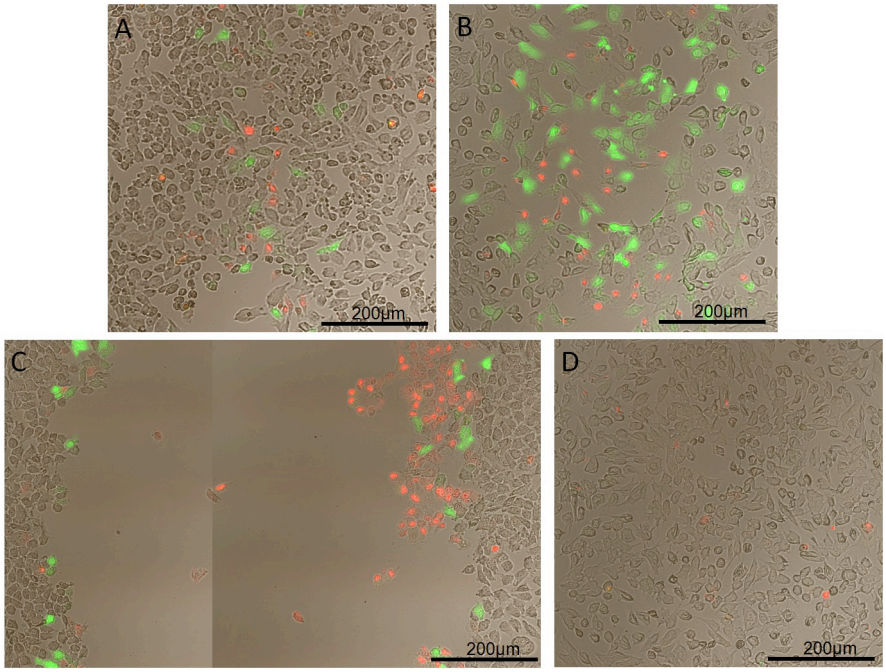

Figure 1: Permeabilization of HeLa cells with 10kDa green fluorescent dextran using a helium gas jet with a capillary nozzle inner diameter of 0.86mm. Dextran fluoresces green and dead cells fluoresce red. A) dynamic pressure of 100 Pa, B) of 135 Pa, C) and of 175Pa D)Control with no exposure to helium gas jet. .

DISCUSSION:

Inert gas jet permeabilization is a useful technique for adherent cell transfection. It provides the ability to transfer biomolecules into cells with the use of mechanical forces which eliminates the need for potentially harmful chemicals or viral vectors. The technique also gives researchers and clinicians an efficient and relatively simple way to precisely transfect cells. The selectivity of the permeabilization is also unique allowing researchers to only treat certain cells in a single colony which could be useful in multiple applications.

Several parameters can be adjusted for individual experimentation such as capillary nozzle diameter, gas flow rate, liquid and capillary height, cell line and target biomolecule to be used. A set liquid level and capillary height have been described in the procedure and these values were chosen to minimize the

number of variables involved in our experiments. Currently, we are investigating the possibility that the shear forces created by the displacement of the liquid are not required to cause permeabilization. This is being done by first removing all liquid from a well, then running the gas jet protocol and immediately after the protocol is completed, adding the permeabilizing solution. Thus far, preliminary trials have suggested that transfection is still occurring under these conditions and the forces of the gas alone may be sufficient.

It is important to take note of the maximum dynamic pressure under which the cells can survive. It was found that once dynamic pressures neared 200Pa for a gas jet nozzle height of 3mm, HeLa cell stripping became likely. This agrees with other studies such as Lu et al who observed WT NR6 fibroblast cell detachment at 200-265Pa[16]. If large amounts of stripping occur, a lower gas flow rate should alleviate this issue. Similarly, high flow rates result in cell death and false-positive permeabilization results. Once cells have died they will become permeable to molecules and therefore dead cells can be mistaken for permeabilized live cells. It is therefore recommended to conduct a live/dead assay to ensure that the permeabilized cells are in fact still alive. Death increases with increasing pressure and our lab found that at 390Pa the majority of permeabilized HeLa cells were dead.

Our hypothesis is that fluid shear stresses on the cells, created by the jet of gas and the displacement of the media, causes temporary disruption of the cell plasma membrane. This is the same mechanism proposed for the other more complex physical methods such as microbubble collapse and sonoporation[17]. The operating conditions for this method are dependent on the type of cells, attachment to the substrate, mechanical properties of the cell and cell-wall, molecule of interest, properties of the liquid and gas, and the physical dimensions of the setup. Through size exclusion studies, we have found that dextran molecules larger than 40kDa show very low permeabilization efficiency and that an optimal dextran size is 10kDa for experimentation. This is important as it limits the size of molecules which can be permeabilized into cells using this protocol.

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DISCLOSURES:

The authors declare they have no competing financial interests.

REAGENTS:

Name of Reagent	Company	Catalogue Number	Comments
Vitality hrGFPII-1	Agilent Technologies Canada	240143-57	Green fluorescent protein for transfection experiments on HeLa cells
Dextran, AlexaFluor® 488; 10,000MW,	Life Technologies Inc.	D22910	dextran for permeabilization

Anionic, Fixable			experiments
LIVE/DEAD® Viability/ Cytotoxicity Kit	Life Technologies Inc.	L3224	for counterstaining of mammalian cells
Prolong® Gold Antifade Reagent	Invitrogen	P36930	for mounting slides when imaging
Ultra High Purity Helium	Praxair Canada Inc.	HE 5.0UH-T	
Hyclone® DMEM/ High Glucose Media- 500mL	Thermo Scientific	SH30022.01	for HeLa cells
Fetal Bovine Serum	Invitrogen	26140079	For DMEM media
Penicillin-Streptomycin, liquid	Invitrogen	15140-122	For DMEM media
PBS 1X			Produced in lab

EQUIPMENT:

Material Name	Company	Catalogue Number	Comments
6-Well Clear TC-Treated Microplates	Corning	3506	
#1 22x22 Coverslips	Fisher	12-542B	
Glass Capillaries	World Precision Instruments		WPI Sizing Information ID (mm) 1.0 0.86 0.68 0.5 Order # 1B200 1B150 1B120 1B100
Mass-Flo Controller	MKS	M100B01314CS1BV	Mass flow controller, Series M100B, requires an external 12V power supply
USB-6009 DAQ Device	National Instruments	779026-01	
UniSlide Assembly with stepping motor and controller	Velmex		Two series MA15 UniSlide Assemblies, fitted with Vexta 1.8° stepping motors provided by Velmex, and controlled by a VXM Stepping Motor Controller

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