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Impact Mediated Loading Cytoplasmic Loading of Macromolecules into Adherent Cells.

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I. INTRODUCTION

The advent of modern molecular biology, including the development of gene array technologies, has resulted in an explosion of information concerning the specific genes activated during normal cellular development, as well as those associated with a variety of pathological conditions. These techniques have served as a highly efficient, broad-based screening approach for those specific genes involved in regulating normal cellular physiology and identifying candidate genes directly associated with the etiology of specific disease states. However, this approach provides information at the transcriptional level only and does not necessarily indicate that the gene in question is in fact translated into a protein, or whether or not post-translational modification of the protein occurs.

The critical importance of post-translational modification (i.e. phosphorylation, glycosylation, sialyation, etc.) to protein function has been recognized with regard to a number of proteins involved in a variety of important disease states. For example, altered glycosylation of beta-amyloid precursor protein results in an increase in the amount of beta-amyloid peptide generated and hence secreted as insoluble extracellular amyloid deposits (Georgopoulou, McLaughlin et al. 2001; Walter, Fluhrer et al. 2001), a pathological hallmark of Alzheimer's disease. Abnormal phosphorylation of synapsin I has been linked to alterations in synaptic vesicle trafficking leading to defective neurotransmission in Huntington's disease (Lievens, Woodman et al. 2002). Altered phosphorylation of the TAU protein involved in microtubule function has been linked to a number of neurodegenative diseases such as Alzheimer's disease (Billingsley and Kincaid 1997; Sanchez, Alvarez-Tallada et al. 2001). Aberrant sialyation of cell surface antigens has been detected in a number of different tumor cell types and has been linked to the acquisition of a neoplastic phenotype (Sell 1990), while improper sialyation of sodium channels in cardiac tissue has been linked to heart failure (Ufret-Vincenty, Baro et al. 2001;

Fozzard and Kyle 2002). In addition to the limitations associated with the total lack of information provided regarding post-translational modification of the encoded protein, gene analysis cannot provide information on the role of protein-protein interactions within the cellular milieu. For example, a combination of gene and protein analysis may indicate that a particular protein is unregulated at both the mRNA and protein levels, respectively. However, the presence of a regulatory protein(s) in the cellular milieu may result in this over-expression being of no physiological importance. For example, cytokine stimulation leading to an elicited cellular response is not only associated with the action of a particular cytokine at its specific receptor, but also with a cascade of down-stream signal transduction events that are under the control of a variety of negative regulatory proteins (Carra, Gerosa et al. 2000; Murphy, Hayes et al. 2000). Such protein-protein interactions underscore the complexity of the inter-play between proteins in modulating cell function and are an excellent example of the limitations of gene analysis techniques in probing "cause-and-effect" relationships between gene expression and cellular response. It is the study of the interaction between gene expression, modified transcript splicing and post-translational modification on protein function as it impacts cellular/tissue phenotype which is now the modern research field of proteomics.

One approach to overcoming the limitations of genetic analysis is to insert a protein of interest into a cell and directly observe the effects on cell function. The direct "insertion" of a protein (in contrast to transfection of a genetic construct followed by protein expression) allows investigation of the functional role of the inserted molecule in determining cell phenotype in a fashion that also considers the role of protein-protein interactions in the cellular milieu. Insertion of individual or mixtures of purified proteins prepared in the **native** state also increases the possibility that any observed effects of the protein on cell phenotype/response are

of true physiological relevance. As yet, such functional proteomic studies are limited by the availability of technologies that allow efficient insertion of native proteins directly into cells.

Micro-injection (Bloom, Evergren et al. 2003; Bubb, Yarmola et al. 2003), electroporation (Chow and Gawler 1999; Ponsaerts, Van den Bosch et al. 2002) and lipid vesicle-mediated protein loading (Chen, Carbone et al. 1993) all have been demonstrated to achieve protein insertion into cultured cells. Mechanical-based loading techniques such as biolistics (Maddelein, Dos Reis et al. 2002), syringe loading (Clarke and McNeil 1992) and scrape loading (McNeil, Muthukrishnan et al. 1989) can also be used to load a variety of cell types with a range of proteins. However, apart from micro-injection which requires expensive equipment and skilled personnel to produce relatively few loaded cells, none of the above techniques have wide applicability to loading of either individual or mixtures of purified proteins into adherent primary cells in tissue culture. We here describe a technique, known as impact-mediated loading, capable of simultaneously loading a large number of adherent primary cells (> 10,000 cells during a single procedure) with a variety of proteins at high efficiency. This approach is based upon the production of transient plasma membrane wounds by particle impact with the cell membrane. We have previously used this technique in order to load dyes, antibodies and plasmid constructs into the cytoplasm of a number of primary and established cell lines utilizing a crude, relatively uncontrolled experimental apparatus (Clarke, Vanderburg et al. 1994). In addition, we have used a more refined version of this technique to study the effects of altered gravitational conditions on the membrane wounding response of human primary myoblasts (Clarke, Vanderburg et al. 2002). Based on observations made during these latter studies, we have developed a novel technology, known as the G-Loader, which utilizes the effects of hyper-gravity at $200xg$ to enhance macromolecular loading into the cytoplasm of adherent cells via impact-mediated plasma membrane wounding (Clarke, Feeback et al. 2001).

The protocol described below specifically details the experimental approach used to load primary human skeletal myocytes with FITC-labeled IgG immunoglobulin as an example of the suitability of the G-Loader technology for loading biologically active proteins into primary adherent cells.

II.   MATERIALS AND INSTRUMENTATION

Dulbecco's modified Eagle's medium – F12 medium (X1 concentration) (DMEM/F12, cat. no. 320-1885AG), bovine fetal calf serum (CS) (cat. no. 200-6170AG), penicillin-streptomycin solution (cat. no. 600-5140AG) are obtained ready to use from Gibco BRL (Grand Island, NY). Alexa Fluor™ 488 labeled goat anti-mouse IgG (Mr – 150,000 kD) (cat. no. A-11001) is obtained from Molecular Probes (Eugene, OR, USA). Tissue culture flasks (T-75, T25 and 35mm diameter plates) (cat. nos. 10-126-41, 10-126-26 and 25050-35), sterile polypropylene conical centrifuge tubes (50 ml capacity) (cat. no. 05-538-55A) are obtained from Fisher Scientific (Pittsburgh, PA, USA). The G-Loader technology and associated consumables where obtained from Peilear Technologies (Houston, Texas). Living cells loaded with fluorescently labeled IgG were examined utilizing an Zeiss Axiophot inverted microscope in order to view them in the 35 mm tissue culture plate directly.

III. PROCEDURES

A.   Preparation of tissue cultured cells for impact-mediated loading using the G-Loader.

Stock Solutions and Media Preparation

1. *Stock IgG solution*

Stock Alexa Fluor™ 488 goat-anti-mouse IgG (2 mg/ml) can be stored at 4°C in the dark for up to a month or aliquoted and frozen in the dark at -80°C for storage up to a year. Prior to use on living cells it is important to remove any preservative (e.g. sodium azide) from the immunoglobulin solution as exposure of living cells to such contaminants can result in rapid cell death. Removal of such preservatives can be carried out by using either a miniature dialysis device or by buffer exchange using a 30,000 MW cut-off centrifugal miniature concentrator. One ml of stock IgG (2 mg/ml) solution should be dialyzed against a minimum of three changes of 100 ml of sterile DMEM/F12 medium over a period of 24 hr prior to use. Total volume of dialyzed IgG solution should be adjusted to original volume prior to dialysis to maintain overall IgG concentration. Buffer exchange using a centrifugal concentrator should involve a minimum of three complete exchanges of the original volume of stock IgG solution with serum-free DMEM/F12 medium.

2. *IgG loading solution*

Add 125 µl of stock IgG solution to 0.875 ml of serum-free DMEM-F12 to obtain final concentrations of 250 ug IgG/ml. Equilibrate this loading solution to correct temperature and pH by incubating in a standard 5% CO_2 tissue culture incubator maintained at 37°C for a period of 30 min prior to loading cells with the IgG.

3. *10% FCS.DMEM/F12 tissue culture medium*

Add 5 ml of sterile penicillin/streptomycin solution and 50 ml of sterile fetal calf serum (CS) to 445 ml of sterile (X1) DMEM-F12 solution, to obtain DMEM/F12 culture medium containing

10% FCS, 100 i.u./ml penicillin and 100 µg/ml streptomycin (10%FCS.DMEM). Store at 4°C for up to 21 days.

B.   Culture of Human Primary Myoblasts

1. Primary human myoblasts are obtained from the Clonetics Corporation (Walkersville, MD) and cultured to confluence in T-75 (75 cm^2) culture flasks using 15 ml of 10%FCS.DMEM/F12 maintained at 37°C in a 5% CO_2 humidified atmosphere with sub-culture every fourth day.

2. The day prior to the loading procedure cells are trypsinized, collected by centrifugation and resuspended in 5 ml of 10%FCS.DMEM/F12 as a single cell suspension. The number of cells in the suspension is determined using a hemacytometer and cell density in the solution is adjusted to 50,000 cells/ml using 10%FCS.DMEM/F12. Two ml of this cell suspension is plated into a 35 mm tissue culture plate and the cells are incubated overnight to allow the formation of a monolayer. Under these culture conditions the center of the 35 mm plate is between 60% and 80% confluent after 24 hr of culture prior to loading.

3. Prior to loading, cells are washed three times over a period of 5 min with warm serum-free DMEM/F12 containing no antibiotics to remove any unattached cells and to wash away serum components that may interact with the IgG molecules being loaded.

C. Impact-mediated Loading of IgG using the G-Loader Technology.

1. Remove serum free medium from the cell monolayer, replace with 400 microliters of

    loading solution and gently agitate the 35 mm plate so that the loading solution covers all of

    the culture surface. Agitate the loading solution for a minimum of 30 sec to ensure that the

    dissolved macromolecule is in contact with the surface of the cells.

2. Remove the loading solution from the 35 mm plate by tilting the plate to one side and

    removing the loading solution with a 1 ml sterile pipette. (N.B. the loading solution can be

    reused on up to five additional 35 mm plates if used immediately after first centrifuging at

    10,000*xg* for 1 min to remove any particulate/cellular material).

3. The cells are now ready to be loaded using the G-Loader technology. Place the 35 mm plate

    in the G-Loader and operate the firing mechanism in the tissue culture hood. Alternatively,

    the device and cells can be placed into the centrifuge and loading performed under hyper-

    gravity conditions (i.e. 200*xg*). In the case of human primary skeletal myoblasts and IgG

    loading, highly efficient cytoplasmic loading of IgG can be achieved without the need for the

    use of hypergravity conditions during impact-mediated loading.

4. Remove the 35 mm plate from the G-Loader and immediately place 2 ml of warm serum

    free DMEM/F12 medium (without antibiotics) into the 35 mm plate to prevent drying of the

    cells. Gently wash the cell layer three times over 2 min with three changes of serum free

    DMEM/F12 medium to remove both excess IgG and the particles impacted with the cell

    surface.

5. Replace washing medium with 10%FCS.DME/F12 tissue culture medium and incubate for the required time period. After this time (0hr – 24hr depending on the goal of the project), wash once with warm D-PBS (pH 7.2), replace with fresh D-PBS and view the living cell monolayer by inverted fluorescent microscopy using UV-excitation and emission at 488nm.

IV. COMMENTS

This technology provides a highly efficient means of loading large macromolecules into living adherent cells with little or no disruption of the adherent monolayer. As can be seen in Figure 1, there is little or no visually discernable damage to the cell monolayer one hour after impact-mediated cytoplasmic loading of a two million Dalton-sized fluorescent dextran. The protocol detailed above describes the procedure required to load fluorescently-labeled IgG into cultured primary skeletal muscle cells (Figure 2). In addition, this technique has been applied to a large number of different cell types and wide variety of different macromolecules, including plasmid DNA constructs as a means of transfecting both primary and established cell lines (Figure 3). Furthermore, this technique has the ability to simultaneously load two or more macromolecules directly into the cell cytoplasm of living cells, which are then localized to specific cellular compartments within the living cell based on size exclusion (Figure 4). In addition, the use of hypergravity conditions during impact mediated loading significantly increases the amount of fluorescently labeled IgG protein loaded into the cell cytoplasm (Figure 5).

V. PITFALLS

The impact-mediated loading technology described here (i.e. The G-Loader) is a highly efficient means of simultaneously loading large numbers of living cells with large macromolecules. Under the conditions required for loading of macromolecules below 1×10^6 daltons in size, the number of cells which are wounded, loaded and survive the procedure is extremely high (approximately 70 – 80% of the starting population) (Figure 1). One of the disadvantages of the technique described above for loading IgG is that not all cells in the 35 mm tissue culture dish are loaded. This is due to a limited particle impact area (approximately one half of the culture area radiating from the center of the plate) generated by the device. This drawback can be overcome by growing cells only in the center region of the 35 mm plates utilizing a 20 mm diameter cloning ring or plating cells on 20 mm diameter glass cover-slips located in the center of the 35 mm plates and removing the cover-slips after loading. This approach yields a cell population in which approximately 70 – 80% of the starting population is loaded. However, in those experiments where direct localization by microscopic techniques is utilized the presence of the unloaded cells in the region closest to the edge of the plate serves as an excellent internal negative control.

A second area of concern is the purity of the macromolecule that is being loaded. In initial experiments using commercially-produced, fluorescently labeled IgG, IgM and plant lectins, impact-mediated loading was observed at high efficiencies immediately after loading as determined by direct inspection of the cell layer by fluorescent microscopy. Subsequently however, the majority of the cells died within two hours of loading. After investigation, it was found that most commercially produced products had some form of preservative in the storage buffer that needed to be removed prior to contact with the living cells during the loading procedure.

The G-Loader technology utilizes a combination of particle impact-induced membrane wounding and hyper-gravity conditions to load macromolecules directly into the cell cytoplasm. This technology appears to have utility for loading in a wide range of cell types, both established cell lines (e.g. COS, 3T3, C2C12 cells) and primary cell types (e.g. human myoblasts, bovine endothelial cells, chick corneal fibroblasts, rat gut epithelium), as well as multi-cellular micro-organisms (e.g. hydra, nematodes, *Xenopus* eggs). However, in order to achieve optimal loading efficiency a mechanistic approach to determining impact pressure, macromolecular concentration in the loading solution and whether or not hyper-gravity conditions are required for efficient loading is suggested. In addition, the nature of the macromolecule being loaded is also important. Those compounds which are highly charged or may interact with cell surface components may need longer contact with the cells prior to loading, a higher loading solution concentration or higher *g*-load (i.e. 200*xg*) (Figure 5) in order to achieve the most efficient cytoplasmic loading possible.

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<u>Figure 1</u> Paired phase-contrast and FITC-fluorescent micrographs of a Swiss 3T3 fibroblast monolayer one hour after impact-mediated cytoplasmic loading of a two million Dalton fluorescent dextran (FDx – 200 kD). The monolayer was washed three times with warm serum-free DMEM to remove excess FDx (200 kD) and to remove the majority of the particles (arrows). Note the clear exclusion of the 200 kD dextran from the nuclear region of the cells (arrow-heads) and little or no visual damage to the cell monolayer (Phase panel). Complete bead removal can be achieved by washing the monolayer multiple times with medium over a period of four hours.
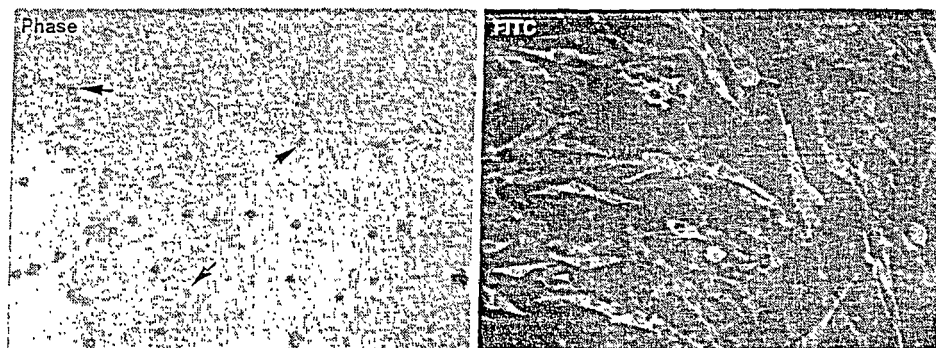
<u>Figure 2</u> Fluorescent micrographs of (A) Control and (B) Alexa Fluor™ 488 goat-anti-mouse IgG loaded primary human myoblasts. Control monolayers are treated in an identical fashion to loaded cells (including incubation with IgG loading solution) except that they are not subjected to impact-mediated loading. Panels A and B were photographed under identical photographic conditions immediately after loading and washing of monolayers. Note the somewhat "perturbed" appearance of the cells, an appearance that disappears after 20 to 60 min of additional culture in serum-containing medium.

Figure 3 Fluorescent micrographs of primary human skeletal myoblasts (HSKMC), Swiss 3T3 cells (3T3) and primary bovine capillary endothelial cells (BCEC) 24 hr after impact-mediated loading of a plasmid construct encoding for a green fluorescent protein, Lantern Green™ (pLG).
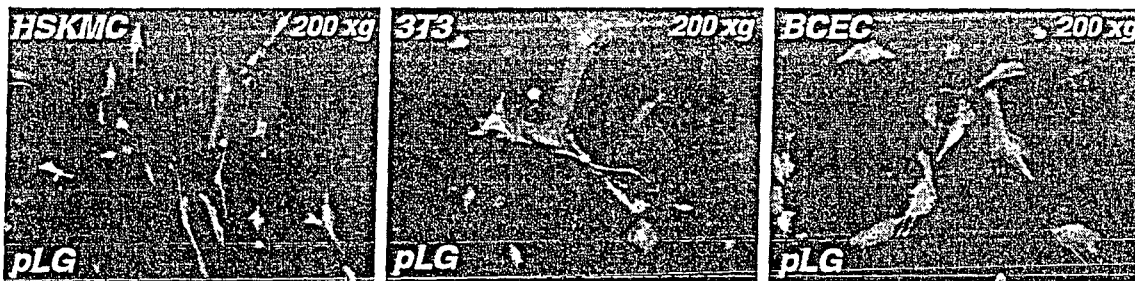
Figure 4 Matched fluorescent micrographs of NGF-differentiated PC-12 cells 20 minutes after impact-mediated loading of a mixture of fluorescently labeled dextrans. Two different-sized, different-colored dextran molecules (*green – FDx -10 kD; red – TRITC- 30 kD*) were simultaneously loaded into monolayers of PC-12 cells using impact mediated loading. Note the exclusion of the larger dextran (red signal) from the nucleus of the cells (Panel B), whereas the smaller dextran (green signal) is found in both the cytoplasm and nucleus of the loaded cells (A). When a digital overlay of the images is generated, cells are colored

predominantly orange in their cytoplasm (i.e. mixed red and green signal), whereas the cell nuclei are green (Panel C).
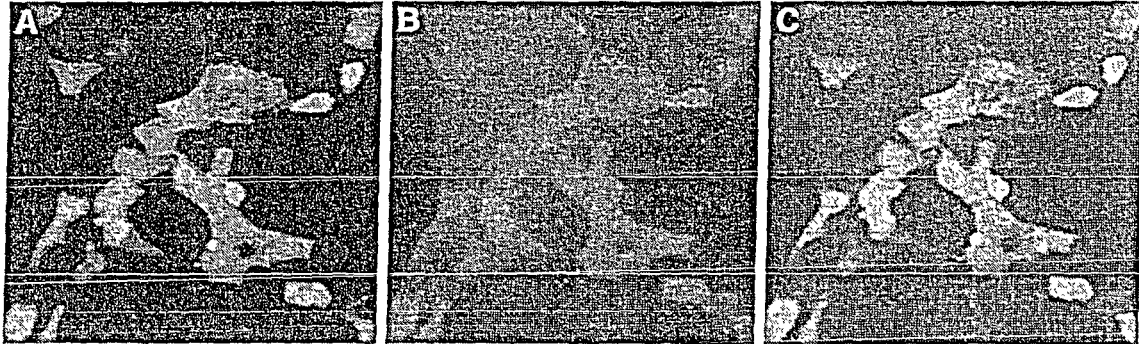

Figure 5 Fluorescent micrographs of Swiss 3T3 cell monolayers cytoplasmically loaded with Fluor™ 488 goat-anti-mouse IgG employing impact-mediated loading at 1xg on the bench-top (Panel B) and at 200xg in a bench top centrifuge (Panel C). Control cells (Panel A) were exposed to IgG for the same period of time but were not impact loaded. Panels A, B and C were taken at the same photographic conditions. Note the larger amounts of IgG present in the cytoplasm of cells loaded at 200xg as compared to those loaded at 1xg and that IgG is excluded from the nuclear regions of the loaded cells.