Perspective

The many ways to cross the plasma membrane

David J. Stephens* and Rainer Pepperkok†

Cell Biophysics and Cell Biology Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

The transfer into living cells of macromolecules, which monitor or modify molecule-specific intracellular processes, provides an efficient way to study the temporal and spatial regulation of protein systems that underlie basic cellular functions. Several methods have been developed for this purpose. Each of them has its characteristic advantages and disadvantages with respect to cell viability, transfer efficiency, general applicability, and technical requirements. We discuss current methodologies for the introduction of macromolecules, notably proteins, into cells in light of a new paper by Walev *et al.* (1) in a recent issue of PNAS in which a new protocol is provided for the reversible permeabilization of cells by using streptolysin-O. The function and interaction of many more novel proteins identified by the genome projects worldwide will have to be analyzed in the future. Therefore, transfer techniques that are easy to use, inexpensive, and suitable for automation such as the one described by Walev *et al.* (1) will become of superior importance.

Worldwide genomic and cDNA sequencing projects are now identifying new molecules every day. The genomic sequences of the most widely studied species, *Saccharomyces cerevisiae, Caenorhabditis elegans*, and *Drosophila melanogaster*, have recently been completed. The full genomic sequence of *Homo sapiens* will become available soon. An enormous task, and a great challenge, will now be to relate these sequences to functional data that may subsequently open new avenues of research toward the cure of diseases. Together with the methodological progress made in the past two decades in expressing and manipulating cloned genes in the test tube, these sequence data provide us with the necessary molecular tools to understand the connectivity and spatial organization of protein systems in their native environment, the living cell.

Common strategies in modern cell and molecular biology, which aim toward the identification of the role of an unknown protein in the context of the intact cell, involve the introduction into living cells of molecules that allow one to monitor the localization or biochemical state of the protein (e.g., by fluorescence microscopy-based methods, ref. 2) or that interfere with its function (e.g., dominant negative mutants). Several such molecule transfer methods have been developed for this purpose. Direct transfer methods introduce the molecule of interest precisely into the cell. In carrier-mediated transfer methods the molecule of interest is loaded into or coupled to a general carrier that can cross the plasma membrane itself and thereby helps the passenger to enter the cell. A third class of transfer methods uses chemicals, bacterial toxins, or electrical pulses to transiently permeabilize the plasma membrane. The molecule transfer occurs then via diffusion through the pores formed.

Each of the methods has its characteristic advantages and disadvantages with respect to cell viability, transfer efficiency, general applicability, and technical requirements (see Table 1).

Direct Transfer Methods

The principal and only widely used direct and most efficient of all transfer methods is glass capillary microinjection, which was first reported about 30 years ago (3, 4). Transfer efficiencies and survival rates of up to 100% can be reached. Glass micropipettes with a fine tip of less than 0.5 μm are used to inject the sample of interest into the cell nucleus or cytoplasm of adherent cells. Microinjection is direct, and quantitative and coinjection of several distinct molecules at fixed stoichiometric ratios is possible. A huge variety of molecules can be injected, and even injection of entire organelles has been reported (5). Furthermore, the molecules of interest can be injected at well-defined stages of the cell cycle, and cell culture conditions can be modified before, during, or after injection. Unfortunately microinjection is technically demanding. Although the specialized equipment needed, including prepulled micropipettes, is now commercially available (see for example ref. 6), a lengthy training period is required until reproducible results are obtained on a routine basis. A further drawback of classical microinjection methodologies is that only a few cells (of the order of 100–200) can be injected in one experiment, and thus analyses by biochemical methods or electron microscopy is difficult. There is also a limitation to the cell types that can be readily used for microinjection. Cultures that grow in suspension are obviously more difficult to use, as are those adherent cells that have only small volume nuclei or cytoplasm. However, computer-automated or semiautomated injection methods allow the injection of up to 1,500 cells per hour and advanced analysis of injected cells has become possible (6, 7). Future developments of such methods certainly will facilitate microinjection further, and it may become possible to even automate the systematic transfer of distinct molecules into a large number of cells at high throughput as would be necessary for large-scale genomic or proteomic projects.

Carrier-Mediated Transfer

In contrast to the direct transfer methods, a vast variety of protocols exist that use a cell-permeable molecule as a generic carrier or to which the molecules to be introduced into cells is coupled to (see Table 1). When the carrier enters the cell the molecule to be introduced enters the cell as well in a "piggyback" manner. Such an approach allows targeting of thousands or millions of cells at the same time, and thus allows, in contrast to transfer by microinjection, much more sophisticated subsequent analyses, such as immunoprecipitation and Western blot analysis, subcellular fractionation, or electron microscopy. Application of the "loaded" carriers to whole animals is possible and can thus be applied in gene therapy approaches. The protocols for carrier-mediated transfer are usually simple, and technically advanced equipment is not required. Several carriers have been developed commercially and are thus readily available. These techniques have, however, been optimized predominantly for transfer of DNA or RNA and are less characterized for their use in transfer of proteins or small cell regulatory molecules that usually cannot cross the plasma membrane.

An interesting naturally occurring carrier is the homeodomain of the *D. melanogaster* protein Antennapedia. This 60-aa peptide is able to enter live cells in an energy-independent process that is different from endocytosis and appears not to require cell surface receptors (8–10). A 16-aa fragment of this peptide (penetra-

*E-mail: david.stephens@embl-heidelberg.de.

†E-mail: pepperko@embl-heidelberg.de.

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Table 1. Common transfer techniques: Advantages and disadvantages

Method	Ref(s).	Advantages	Disadvantages
Direct transfer			
Capillary microinjection	6	High efficiency (100%) and survival rate, quantitative, few restrictions on target cells and sample, compartment specific transfer	Limited number of cells can be injected, expensive and technically demanding
Carrier-mediated transfer			
Protein transduction	11	Receptor, energy and transport independent, simultaneous transfer to large number of cells, *in vivo* applications possible	Size restriction in molecules that can be transferred, transfer is accompanied by protein unfolding
Liposome-mediated transfer	18	High efficiency, little restrictions in sample size, variety of molecules can be transferred, *in vivo* applications possible	Interferes with lipid metabolism
Microprojectile bombardment	22, 38	Applicable to wide range of cells (including plant cells), surface receptor independent, excellent for *in vivo* gene therapy	Specialized equipment needed, works best for DNA transfection
Transfer by plasma membrane permeabilization			
Reconstituted systems	26, 28, 30	Exchange of whole cytosol possible, large number of target cells addressable, cheap and simple to use	Cells are semi-intact
SLO permeabilization	1	Works with variety of sample molecules, high efficiency, cells remain intact, large number of target cells addressable, cheap and simple to use, could be automated	Size restriction (100 kDa) of molecules that can be transferred
Electroporation	35, 36	Large number of target cells addressable, applicable to a wide range of cells (including plant cells), little sample restrictions, high efficiency	Requires specialized equipment, works best when cells are in suspension

tin-1), which is short enough to be easily synthetically synthesized, has been shown to retain the properties necessary for penetration. Unfortunately, attempts to routinely introduce large proteins or DNA molecules (>100 residues) have been difficult so far (11, 12). The penetratin system is also ineffective for the uptake of double-stranded DNA (8).

A further commercially available method is based on the VP22 protein of herpes simplex virus 1. The VP22 protein has a unique ability to translocate between mammalian cells. Expression of VP22 fusions in cells facilitates expression of a fusion protein that is exported from the transfected cells and is then translocated (transduced) into the nontransfected cells where it localizes to the nucleus (e.g., see ref. 13); however, the originally transfected cells show a principle localization of the fusion proteins to the cytoplasm. One must consider the localization of the proteins used in such experiments in relation to biological activity. It is possible to provide a homogenous population of cells by preparing cell lysates from the originally transfected cells and incubating this with a separate population. Of particular note are a number of recent publications concerning the transfer of VP22-GFP (green fluorescent protein) fusion proteins between cells (13–15). These studies consistently report difficulties in the detection of GFP fluorescence in cells to which the VP22-GFP fusion has been transferred (as opposed to the original, transfected cells); GFP fluorescence was not detectable in living cells and only weakly so when fixed (13–15). Although this is possibly due to the folding status of GFP, pH-dependent quenching, or some other form of quenching, it seems most likely that transfer of the protein was simply below the level of detection in these experiments (13).

A similar technology to VP22 is the use of the TAT protein from HIV-1. The TAT protein transduction domain is typically fused to the protein of interest and generated as a recombinant protein in bacteria (see ref. 11). After purification, the fusion proteins are used to transduce cells with high efficiency. The key limitation of this approach in terms of biological delivery of active proteins is the requirement for the partial or even complete unfolding of the protein that occurs during the transduction process (11). Despite this, many active enzymes have been successfully delivered as TAT fusions (see ref. 11). This approach, like that using VP22, has the benefit of being able to deliver large protein molecules of over 100 kDa. Indeed, 40-nm supraparamagnetic iron particles were taken up efficiently when coupled to multiple copies of the TAT peptide sequence (16). The technique is also applicable to every eukaryotic cell line tested thus far with the exception of yeast. Even *Drosophila* has been shown to be susceptible by painting the eyes with a solution containing the fusion protein (11). The key to this technology would appear to be the refolding of the TAT fusion proteins within the cells.

Other more universal approaches of carrier-mediated transfer, which also allow transfer of larger molecules, use cationic lipids for introduction of DNA, RNA, or proteins into cells (17, 18). Most protocols use commercially available monocationic lipids such as DOTAP (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate), DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride), or DOSPA (2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate). In particular, success has been achieved with some of the newer formulations of these lipids (17). Unilamellar or multilamellar liposomes with a diameter between 100 and 400 nm are generated. The positive charges of the liposomes allow them to interact spontaneously with the negatively charged backbones of polynucleotides to form complexes. These can then interact with the negatively charged cell membrane, resulting in the delivery of the polynucleotides into the target cells. This approach eliminates the need for the generation of fusion proteins or chemical peptide protein coupling but is likely limited to acidic proteins that complex readily with the cationic lipids routinely used. Because the ability of these liposomes to incorporate water- or lipid-soluble molecules, liposomes also have

been used as a means to deliver pharmacologically active molecules to specific sites in the body (17, 19, 20). Liposome-mediated delivery needs to be interpreted with caution because significant changes in cellular metabolism can be induced, particularly with regard to lipid metabolism (21). A new generation of liposome-like transfection reagents has started appearing on the market recently. The Chariot reagent from Active Motif (Carlsbad, CA) is apparently specifically designed for protein transfection and boasts efficiencies of 60–85%. Such technologies are clearly a welcome development but await wider usage and independent testing before a thorough evaluation of their potential.

Introduction of probes into cells or tissues by microprojectile bombardment uses subcellular-sized particles, which are coated with DNA and accelerated at high velocities toward target cells (22, 38). Thousands of cells can be penetrated and transformed with one bombardment. The physical nature of the process makes it applicable to a wide range of cell and tissue types and a diversity of organisms. It has thus become one of the most widely used methods for plant transformation for which many other transfer methods fail because of the rigid nature of the cell wall. More recently, this method has been applied to deliver DNA to whole tissue *in vivo* (23) and thus represents a true alternative to viral vectors in gene therapy (24).

A major uncertainty in most carrier-mediated transfer methods is that efficiency varies with cell type (18) and usually is far below 100%. This poses problems in the interpretation of those experiments where the molecule introduced has inhibitory function. With a typical transfer efficiency of for example 30%, the maximum inhibition for the entire cell population analyzed will approach 30%, and thus the significance of results is unclear unless the transfer efficiency can be precisely determined independently. Enrichment of successfully targeted cells, by, for example, flow cytometry, may help to overcome such problems.

Transfer by Transient Permeabilization of the Plasma Membrane

A third class of transfer methods uses detergents, pore-forming toxins, UV laser illumination, or short electrical pulses to permeabilize the plasma membrane. Transfer then occurs via passive diffusion through the pores. A critical parameter in these approaches is the survival rate of the cells. Experimental conditions, which generate sufficiently large pores for sufficient molecule transfer, coincide with increased cell death in a fraction of the cells. On the other hand, in the same experiment some cells are often not sufficiently permeabilized and thus the number of successfully permeabilized and vital cells is around 50% of the entire target cell population. Similar to the carrier-mediated transfer techniques, this poses a problem when inhibitory molecules are to be introduced.

Although it has been difficult to overcome these limitations, cell permeabilization by pore-forming toxins or detergents has been extensively used in the past in so-called ''reconstituted systems'' using ''semi-intact'' cells. In these methods cells are irreversibly permeabilized, which even allows exchange of the entire cytosol of the cells (25–30). This has clear advantages because the exogenously added cytosol can be immuno-depleted of interesting factors and the molecules to be introduced (e.g., mutants of the molecule of interest) are thus not in competition with the endogenous pool of the molecules of interest. Addition of exogenous components to assays have been extremely useful for studying many systems, for example, the recruitment of protein complexes to membranes. The ability to detect only the exogenously added, purified adaptor complexes against a background of endogenous protein greatly facilitated analysis of adaptor recruitment to membranes (31). Species-specific antibodies against bovine adaptors enabled specific detection in recruitment assays using rat cell lines (31). The major concern in using these methods remains that it has always been difficult to assess to which extent the semi-intact cells are a valid model for the intact cell. Clearly these approaches are an excellent step toward dissecting molecular processes; however, there is always a concern that these detergents also affect important cellular structures, such as the cytoskeleton, or membrane organelles and therefore semi-intact cell systems are by no means perfect models for the living cells.

Considerable progress has now been made to overcome these limitations by recent work using the pore-forming toxin streptolysin-O (SL-O) (1, 32–34). Under certain conditions cells can repair SL-O lesions (32, 33) and fluorescent proteins could be demonstrated to be taken up by cells treated with low doses of SL-O. Walev *et al.* (1) have succeeded now to provide very good evidence that proteins of up to 100 kDa can be taken up by SL-O-permeabilized cells without any loss of their function. The protocols developed allow about 50% of the treated cells to take up the molecule of interest and to survive for days without any obvious sign of lethality. Cell sorting may indeed be a suitable step to enrich for those cells that have taken up the molecules used. A key point to note from these experiments, as Walev *et al.* (1) note themselves, is the purity of the SL-O used for the studies. Furthermore, accurate estimation of cell permeabilization and recovery is essential to accurate interpretation of results. As with many of the carrier-mediated protocols described above, transient SL-O permeabilization was found to have an effective size limit in terms of efficient uptake. Although small proteins such as antibody Fab fragments and low molecular weight dextrans were efficiently taken up, Walev *et al.* found that molecules above ≈100 kDa were excluded from the permeabilized cells (whole molecular antibodies and high M_r dextrans). This finding is in a similar range to the majority of experiments using carrier-mediated methods.

Similar features to those obtained with these new developments also can be achieved by electroporation, which uses microsecond, high-voltage pulses to transiently permeabilize the plasma membrane (35, 36). Similar to the SL-O method about 50% of the treated cells survive and take up the molecule of interest. Unfortunately, in most applications cells need to be in solution for electroporation, which can be achieved by treatment with trypsin or chelators of metal ions in the case of adherent tissue culture cells. This, however, disrupts a number of important cellular relationships as cell cycle progression, cell adherence, and signal transduction, which are therefore difficult to be studied with electroporation.

As with all of the methods described here there are experiments that clearly benefit from one or another of these approaches in particular. For example, a quantitative study of delivery of antisense oligonucleotides to chronic myeloid leukaemia cells showed SL-O permeabilization or electroporation to be superior to liposome-mediated delivery (37). Indeed electroporation was found to give more reproducible results than SL-O treatment, suggesting great variability in the efficacy of SL-O within individual experiments.

It is clear that further developments of this approach will be necessary in the future, but the method as it stands so far appears to combine the advantages of the semi-intact strategies as simplicity, possibility to exchange cytosolic factors with a high degree of surviving cells. This new protocol now allows exchange of cytosolic factors on a large scale by simple treatment of cells under conditions that keep cells intact.

Conclusions

None of the transfer techniques described above are of sufficient flexibility to cover all possible applications and demands of molecule transfer into living cells or tissues. Future developments should include, however, possibilities for automation of the methods to reach the throughput required for systematic large-scale genome projects. In this respect the method described by Walev *et al.* (1) may have great potential as it requires easily automated handling steps, only pipetting and washing of reagents and cells, and

does not require specialized equipment. In addition, it offers acceptable transfer efficiencies and most importantly permeabilized cells appear to remain intact. Increasing the throughput in capillary microinjection, which is so far unmatched in its transfer efficiency and flexibility in the kind of molecules that can be transferred, by further automization of the method may be another route to take.

As important as the improvements in terms of throughput will be to improve the possibilities to deliver genes, small molecules, drugs, or even proteins into tissues of whole bodies, because transfer of molecules *in vivo* will be of critical importance to translate the forthcoming wealth of information on individual genes by genome projects into animal models and therapies.

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