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EFFICIENT AND INNOCUOUS LIVE-CELL DELIVERY: MAKING MEMBRANE BARRIERS DISAPPEAR TO ENABLE CELLULAR BIOCHEMISTRY::

How better cellular delivery tools can contribute to precise and quantitative cell-biology assays

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Abstract

The confluence of protein engineering techniques and delivery protocols are providing new opportunities in cell biology. In particular, techniques that render the membrane of cells transiently permeable make the introduction of non-genetically encodable macromolecular probes into cells possible. This, in turn, can enable the monitoring of intracellular processes in ways that can be both precise and quantitative, ushering an area that one may envision as cellular biochemistry. Herein, I review pioneering examples of such new cell-based assays, provide evidence that challenges the paradigm that cell penetration is a necessarily damaging and stressful event for cells, and highlight some of the challenges that should be addressed to fully unlock the potential of this nascent field.

Introduction

Let's assume that one can deliver macromolecules into the cytoplasm and nucleus of human cells efficiently and without causing cellular damage. One can then imagine that a multitude of exciting applications would become possible. In particular, the study of protein function may extend beyond what is currently possible with genetically encodable systems. Specifically, several protein engineering technologies can now be routinely used to produce proteins that contain moieties other than natural amino acids. These moieties include spectroscopic probes, post-translational modifications, or photo-activatable switches that can control where a protein goes or what it does. Their incorporation into proteins often requires synthetic steps that must take place using purified components. The semi-synthetic proteins produced have proven useful in biochemical and biophysical in vitro studies. However, introducing molecules into cells remains challenging and these reagents have not yet found broad applications in cell biology. Several pioneering studies however point to what may soon become possible. Herein, I highlight several recent examples of innovative cell-based assays, discuss the developments in cell delivery that are making these assays possible, and

Conflict of interest

The author declares no conflict of interest.

highlight the hurdles that remain to be addressed to reach the full potential of these approaches.

1. Progress in cell delivery is driving the development of new cell-based assays.

1.1 Protein structure—The structure of proteins is determined by X-ray crystallography, cryogenic electron microscopy, or nuclear magnetic resonance (NMR) spectroscopy. These approaches use purified proteins that are either crystallized, embedded in vitreous water, or suspended in buffers, respectively. These techniques therefore provide snapshots of the conformations that proteins adopt in environments that may or may not resemble the interior of a cell. In particular, the cytoplasm is known to be viscous when compared to aqueous solutions, soluble proteins navigating within a crowded space and continually interacting with their neighbors. Conceptually, these effects are likely to impact protein structures and it would consequently be valuable to monitor the conformations taken by proteins directly in the cellular environment. Inomata *et al.* demonstrated that an isotopically-labeled ubiquitin, obtained from a bacterial expression system supplemented with ^{15}N -labeled amino acids, could be purified *in vitro* and introduced into human cells using a protein transduction domain combined with a small molecule acting as an enhancer of cell penetration (Figure 1).^[1] Upon delivery, the ubiquitin protein distributes within the cytosolic space of cells, surrounded by interacting partners that the protein would naturally interact with. Because it is the only species enriched with ^{15}N nuclei, the protein is specifically detected by nuclear magnetic resonance. In turn, this approach permits the determination of protein structure and protein dynamics *in situ*, as opposed to *in vitro*. Recently, Hikone *et al.* have expanded on these ideas by introducing a ubiquitin labeled with a lanthanoid-chelation tag into cells by electroporation.^[2] The authors successfully used this approach to perform pseudocontact chemical shifts measurements and assess the effects of intracellular crowding on protein structure.

1.2 Function of post-translationally modified proteins—Post-translational modifications (PTMs) greatly amplify the complexity of protein species present inside human cells. Establishing how PTMs impact protein function is a vibrant area of cell biology. Molecular biology techniques do not typically permit the controlled production of chemically defined post-translationally modified proteins in a cell. It is therefore challenging to establish how a modified protein differs from its unmodified counterpart. On the other hand, protein engineering techniques can be used to introduce post-translational modifications (PTMs) at specific sites of a protein sequence. To date, these approaches have been successfully used to probe how PTMs impact protein function *in vitro*. Several studies, using ubiquitin as a model have recently highlighted how these approaches can extend to studying PTMs directly within a cell. A synthetic ubiquitin labeled with a fluorophore was delivered into cells.^[3] This PTM substrate was incorporated into intracellular proteins by the ubiquitination machinery. The authors propose to use this system to monitor ubiquitination in real-time. Semi-synthetic ubiquitin labeled with electrophilic groups at its C-terminus have also been recently delivered into cells.^[4] In this case, these constructs can be used to capture proteins involved in the processing of this PTM. In turn, this may be used to identify deubiquitinases, enzymes that remove ubiquitin from modified proteins, and screen for small molecule drugs that alter this process. Finally, trans-

splicing has been used to modify the histone H2A with ubiquitin directly into the nucleus of cells.^[5] This approach relies on the pairing between a synthetic fragment administered extracellularly and a genetically-encoded fragment expressed in the cell. The selective combining of the two protein fragments is made possible by the use of two complimentary halves of a split-intein. One can therefore introduce desired ubiquitin PTMs into histones in cellular nucleosomes (Figure 1). Conceptually, one can envision expanding this approach to the many PTMs that modify histones. Alternatively, fully synthetic histones modified by PTMs using several of the chemistry-based labeling strategy currently available could be delivered into cells directly, assuming proper integration into nucleosomes post-delivery.^[6] Alternatively, the nuclease-deficient Cas9 (dCas9) can be modified synthetically to display chemical or proteinaceous epigenetic probes.^[7] Upon complex formation between a guide RNA and upon subsequent successful delivery into live cells, such constructs are directed to specific genomic localizations. This approach can be used to decorate chromatin with various functionalities or induce the recruitment of histone modifying enzymes. Overall, such strategies are complementary and should enable the probing of the mechanisms of epigenetic chromatin regulation *in situ*.

1.3 Protein-Protein interactions—What proteins do is intimately related to what they bind to. Consequently, identifying protein-protein interactomes, the set of all the molecular interactions taking place in a cell, is key to deciphering protein function. In principle, delivering proteins into cells may complement approaches currently available for interrogating interactomes. A cell delivery approach that would consist of introducing protein labeled with proximity probes may be advantageous as it would enable the probing of interactions *in situ*, thereby alleviate artifact that can be obtain when using techniques relying on cell lysates. Because cell delivery may in principle allow the introduction of a protein into a cell both rapidly and with control over concentration, such a strategy would also circumvent artifacts that could arise when instead overexpressing a protein probe over a period of several hours.

To detect binding events in a cell, proximity probes such as photocrosslinkers can be incorporated into a protein structures. Photocrosslinkers are molecules that become radicals upon light irradiation and that subsequently form covalent bonds with moieties in their direct vicinity.^[8] Binding partners can therefore become linked to one another and retrieved by addition of an affinity tag. Alternatively, with the development of labeling enzymes such as APEX or biotin ligase, biotin tags can be “sprayed” on proteins that are on the direct vicinity of an interaction probe.^[9] Futaki and co-workers have labeled cell-penetrating polyarginine peptides with a photocrosslinker to assess what the peptide interacts with outside and inside the cell.^[10] It is easy to imagine how this approach could be expanded to other macromolecules (Figure 1). For instance, Muir and co-workers have proposed delivering dCas9 labeled with crosslinkers into live cells as a means to probe for the protein content of specific genomic loci (dCas9 being target to specific DNA sequences as described in 1.2).^[7] Such an approach could help reveal new chromatin-modifying protein complexes. One can also envision how proteins modified with PTMs and affinity probes could be delivered into cells to probe how individual PTMs modulate a protein’s interactome. Moreover, given that delivery protocol can, in principle, allow the control of the concentration of protein that

enters cells, it may be possible to perform cell-based titration assays akin to what is typically done *in vitro* for the determination of binding constants of purified protein systems. Thus, it may be possible to identify complex networks of interacting partners while also establishing the relative affinity of each interaction *in situ*.

2. What are some of the challenges involved in making cell delivery approaches work for cell biology assays?

2.1 Maximizing Delivery Efficiency—A plethora of strategies have been pursued to achieve cell delivery, as highlighted in extensive recent reviews.^[11, 12] Because this article focuses on assays that probe the intracellular biochemistry of individual cells, techniques that are pertinent to tissue cultures using either cell lines or primary cells are highlighted in this section. However, it is important to note that many delivery strategies have been developed for *in vivo* applications.^[13] While these applications often have a therapeutic purpose, they may also include cellular probing (not discussed herein).

One general delivery approach consists of puncturing the plasma membrane of cells. This can involve mechanical tools that pierce through lipid bilayers. An example of such approach is microinjection, a technique consisting of using a microscopic needle to can inject small volumes of material into a cell. Microinjection has been available and routinely used for several decades. However, because injections are performed manually in only few cells at a time, this technique limits the type of cell-based assays that can be performed, favoring for instance single-cell fluorescence microscopy over bulk analyses. New developments in material science and nanotechnologies are however enabling the injection of thousand of cells at once. In particular, devices that containing arrays of microscopic needles can be pressed onto a culture to puncture a large number of cells simultaneously.^[14] Electroporation is a delivery approach that can also be used to permeabilize many cells as once. The efficiency of this process, which relies on inducing the formation of membrane pores transiently by application of an electrophoretic field, is often high and adequate for a variety of cell-based assays. However, this delivery approach is often stressful to cells, opening pores not only in the plasma membrane but the membrane of other organelles as well. Electroporated cells therefore often require a recovery time before they can return to a relatively healthy state. A recent solution to this problem involves growing adherent cells onto surfaces containing millions of nanostraws pointing upward.^[15] These nanostraws can then puncture cells upon activation of an electric current that favors pore formation at the site of contact between straws and cells. Macromolecules flowing from a fluidic device into the straws can then enter cells. The coincidence between the site of pore formation and fluid delivery enhances the delivery efficiency over standard electroporation, and, in principle, may allow for shorter time of recovery for cells as the membrane damage performed is more localized. Alternatively, cells can be passed through microfluidic devices that constrict cells. This “squeezing” causes transient membrane deformation and allows macromolecules present in the incubation media to enter cells.^[16] Delivery efficiencies obtained with this technique are comparable to those obtained with electroporation. A notable difference, however, is observable in how cells respond to these treatments, microfluidic squeezing being markedly less disruptive to cells. In particular, genome-wide profiling shows that 34%

of genes are misexpressed 6h after electroporation of T cells (8,141 transcripts out of 23,786 probed) while only 9% are affected 6h after squeezing (2,211/23,766).^[17]

Chemical reagents can be a practical way to introduce macromolecules into live cells. In particular, delivery reagents do not require specialized expertise or instrumentation, thereby facilitating adoption by most research laboratories. For several decades, most chemical agents have been limited by the problem of endosomal entrapment. For instance, when observing by microscopy cells incubated with fluorescent macromolecules conjugated to many commercially available reagents, one typically observes a punctate distribution within cells. These puncta correspond to endosomes filled with the fluorescent material. On one hand, this indicates that the material is indeed successfully internalized by the cell following endocytic uptake. On the other hand, it also indicates that most of the molecules delivered remain trapped in the lumen of endosomes, unable to reach the cytosolic space, the nucleus, or other organelles. Notably, a few molecules certainly reach these locations. There may not be enough to provide a fluorescence signal but they can be detected by assays that rely on signal amplification (i.e. downstream gene expression of reporter proteins). Yet, while low efficiency of cytosolic or nuclear delivery may be sufficient to achieve a biological effect when delivering nucleic acids or enzymes, it is often inadequate when considering assays as those presented in Section 1. Recently, several reagents that can mediate endosomal escape and, thereby improve cytosolic delivery, have been developed. One example, developed in our laboratory, is dfTAT.^[18] dfTAT is a dimeric analog of the widely used CPP TAT. TAT typically leads to extensive endosomal entrapment. In contrast, dfTAT is capable of specifically permeabilizing late endosomes, releasing macromolecular cargos into the cytosol effectively. In particular, dfTAT achieved successful cytosolic delivery of macromolecules in most cells in a culture, while leaving little material trapped in endosomes. dfTAT successfully delivers small molecules, peptides, proteins and nanoparticles.^[18, 19] Several reagents with similar endosomal permeabilization activities have also been reported.^[20] These reagents permit delivery through simple co-incubation protocol and do not require the modification or labeling of the delivered macromolecules. In turn, this means that the function of the macromolecules delivered is not altered once cytosolic delivery is achieved. Moreover, by adjusting the amount of material present in incubation media, such co-incubation protocols allow for the relative control of the concentrations of macromolecules delivered in the cytosol.^[18]

2.2 Minimize Cellular damage.—A concern that arises when using delivery techniques for cell biology studies is that of cellular damage. For instance, probing protein function directly into cells is presumably only meaningful if the cell itself is relatively unperturbed. In a worst-case scenario, delivery is accompanied by cell death, a consequence of extensive damage to cellular membranes. In less dramatic and less visible instances, treated cells may experience various levels of stress and become significantly different than untreated cells. Several studies have recently highlighted how cells respond to delivery reagents. Delivery reagents that perturb the plasma membrane or the membrane of endosomes can for instance promote the influx of calcium into the cytosol (Figure 2).^[21] If a relatively large amount of calcium enters the cytoplasm, and if this influx is rapid enough to overwhelm calcium pumps that would otherwise reestablish homeostasis, cells will undergo necrosis or

apoptosis. In less severe cases, calcium influx triggers the recruitment of repair proteins, including Annexin2 or ESCRT, to damage membrane sites.^[22] Membrane repair may then involve membrane patching by recruitment and fusion of internal vesicles or by the exocytosis of lysosomes at the plasma membrane. Alternatively, cells may also shed vesicles bearing membrane wounds into the extracellular milieu, or, concomitantly, sort these vesicles within the endocytic pathway and target them for degradation. Membrane damage can also lead to the cytosolic exposure of molecules otherwise restricted topologically to the exterior of cells. For instance, proteoglycans located in the lumen of endosomes become accessible to cytosolic galectins upon membrane disruption.^[23] The accumulation of galectins on the surface of disrupted endosomes appears to trigger the subsequent degradation of the damaged organelles by autophagy.

In principle, one may expect that the higher the delivery efficiency, the greater the damage to membranes, and the greater the stress imposed onto cells. Delivering cellular probes efficiently into cells without perturbing them may therefore be an unreachable goal. Routine protocols such as DNA transfection would certainly support this notion, as the exposure of cells to transfection reagents such as Lipofectamine can induce substantial changes in cellular gene expression profiles.^[24] Yet, the delivery agent dFTAT induces a minor gene expression response, 11 mRNA transcripts being misregulated out of 47,000 transcripts probed, as measured after incubating cells for 1h with 5 μ M of reagent.^[18] Moreover, the gene expression of treated cells is virtually undistinguishable from untreated cells 1h post-incubation. This is surprising because microscopy assays suggest that the vast majority of late endosomes within cells have been permeabilized by the endosomolytic agent under these conditions. Considering that a given cell may contain tens to hundreds of late endosomes, and given that very large macromolecules can escape late endosomes as a result of dFTAT-mediated membrane permeabilization, the level of membrane disruption achieved is quite significant and therefore presumably conducive to rather dramatic gene expression changes in the cell. A possibility is that dFTAT works through a somewhat unique membrane-disruption mechanism that is traceless to cells. In particular, because late endosomes are multivesicular in nature, it is possible that membrane translocation events take place within the lumen of endosomes, away from the cytoplasm, and therefore undetected by repair mechanisms. Another possibility is that the membrane repair mechanisms described above mask the deleterious effect that cell penetration might have. In particular, given that the integrity of membrane is essential to cell survival, the expression of proteins of the membrane repair machinery may be constitutive and independent of stimulation by membrane damage. If true, why would dFTAT and Lipofectamine be different in how they induce a gene expression response? A possible answer to this question may reside in the fact that dFTAT is readily degraded by cells. Specifically, D-dFTAT, a dFTAT analog made of D-amino acids instead of L residues, is equivalent to dFTAT in its cell penetration activity.^[25] However, while dFTAT is degraded within minutes upon entry into cells, D-dFTAT is resistant to proteolytic degradation and remains intact inside cells for several days. Notably, unlike dFTAT, D-dFTAT inhibits cell proliferation and leads to the dysregulation of hundreds of genes upon cell delivery. It is therefore possible that, like D-dFTAT, a reagent such as Lipofectamine, which contains unnatural cationic lipid components that are presumably not readily degraded by cells, exert some of its cellular response because

of its long retention inside cells. More importantly, Lipofectamine-like reagents often display relatively poor membrane penetration activity, as evidenced by the fact that complexes between nucleic acid and cationic lipids, primarily localize inside endosomes.^[26] Overall, this emphasizes that membrane penetration efficiency and cellular perturbations are not necessarily correlated. On one hand, low efficiency reagents can certainly have deleterious effects. On the other hand, it also means that high efficiency delivery reagents can be relatively innocuous, supporting the notion that delivery conditions that favor cell biology assays in unperturbed cells may be achievable.

2.3 Protecting Probes From Degradation—Cell-permeable small molecules that can diffuse and equilibrate across membranes reach steady concentrations in cells for extended period of time. Similarly, transient DNA transfection leads to the sustained expression of gene products for a period of several days. The cellular delivery of macromolecular probes into cells is fundamentally different. In particular, macromolecules such as proteins are typically introduced into a cell in an event that involves transient cell permeation (as continuous permeation would most likely enhance the deleterious responses described above). Such probes are then immediately subjugated to degradation pathways (they may also degrade during the delivery process itself). The delivery process therefore typically results in concentration pulse of material into cells. The intracellular concentration of the delivered material will then decline, the half-life of a probe being intrinsic to the probe itself. Depending on the probe and on the assay involved, investigators may then be confronted to a race against the degradation clock. Let's take a live cell NMR application as an example. NMR acquisition time can be on the order of hours. Ubiquitin is relatively stable over this time period and its *in situ* NMR spectra could be successfully acquired. Such experiments would however not be possible for proteins with very short half-lives. Some of the consequences of degradation may also be worse than simply losing signal. In particular, if degradation is not complete but instead partial, one can envision how a protein may for instance be cleaved into various fragments. These fragments could then act as dominant-negative products that, in turn, antagonize the function of the parent protein. Therefore, if we now consider the delivery of a protein-protein interaction probe, partial degradation of the probe may lead to the identification of erroneous interacting partners.

Conclusions and outlook

Progress in the area of cell delivery has been rapid in the last few years. In particular, improvements have been achieved in efficiency and in the variety of molecules that can be delivered. In turn, it is reasonable to expect that further refinements are possible, especially given that the increasing number of mechanistic insights gained in this field should facilitate the fine-tuning of rationally designed tools. The continuous development of delivery approaches is also motivated by highly desirable biotechnological and therapeutic applications. For instance, RNA interference, gene editing with CRISPR-Cas9 or iPSC reprogramming are all applications that require the transient delivery of macromolecule into live cells.^[12] Overall, one can therefore be optimistic that cell delivery approaches will continue to improve. As pointed out herein, this could then tremendously benefit the field of cell biology, leading to a variety of assays that permit the probing of macromolecular

function in a manner that permits the quantitative control of standard in vitro biochemical assays while, in contrast to the often inadequate test tube, providing the biological relevance of the cellular environment.

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Figure 1.

Examples of cell biology assays enabled by the combination of protein engineering and cell delivery approaches. The structures used for this figure are PDB-3KWQ and PDB-3ONS

Figure 2.

Models of cellular responses induced by membrane permeation. The membrane repair and degradation processes described have been detected or postulated for cell delivery agents and for a variety of other membrane-disrupting agents, including toxins, viruses, bacteria, particles, or small molecule drugs.