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## Trapped! A Critical Evaluation of Methods for Measuring Total Cellular Uptake versus Cytosolic Localization

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### Abstract

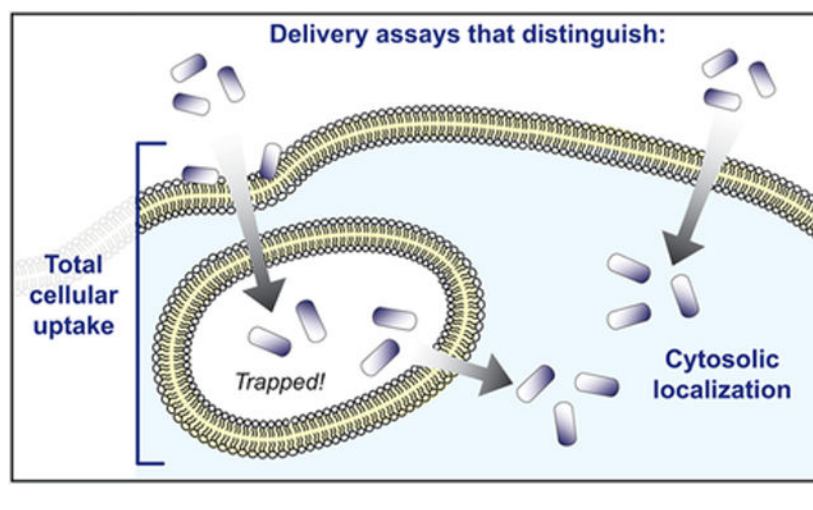
Biomolecules have many properties that make them promising for intracellular therapeutic applications, but delivery remains a key challenge because large biomolecules cannot easily enter the cytosol. Furthermore, quantification of total intracellular versus cytosolic concentrations remains demanding, and the determination of delivery efficiency is thus not straightforward. In this review, we discuss strategies for delivering biomolecules into the cytosol and briefly summarize the mechanisms of uptake for these systems. We then describe commonly used methods to measure total cellular uptake and, more selectively, cytosolic localization, and discuss the major advantages and drawbacks of each method. We critically evaluate methods of measuring “cell penetration” that do not adequately distinguish total cellular uptake and cytosolic localization, which often lead to inaccurate interpretations of a molecule’s cytosolic localization. Finally, we summarize the properties and components of each method, including the main caveats of each, to allow for informed decisions about method selection for specific applications. When applied correctly and interpreted carefully, methods for quantifying cytosolic localization offer valuable insight into the bioactivity of biomolecules and potentially the prospects for their eventual development into therapeutics.

### Graphical Abstract

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## INTRODUCTION

The majority of successful biotherapeutics target extracellular receptors because efficient intracellular delivery is a challenge. Small, sufficiently hydrophobic molecules can cross the plasma membrane without hindrance, but most biological macromolecules, which are much larger and more polar, cannot. Intracellular delivery of macromolecules has been the subject of intense research for many years, ever since the observation of specialized translocation domains that permit cellular transduction. The allure of using biomolecules such as proteins, peptides, and nucleic acids as therapies has increased interest in intracellular delivery strategies. Here, we discuss these advances with a critical eye toward methods used to quantitate intracellular delivery.

At the outset of any discussion of cell penetration, it is essential to clarify the concepts of “inside” and “outside” the cell. A eukaryotic cell is surrounded by a plasma membrane, which routinely engulfs extracellular molecules in a process called endocytosis.<sup>1,2</sup> Importantly, after endocytosis, the macromolecule is still topologically “outside” the cell, as it remains trapped in a vesicle and separated from the cytosol by a plasma membrane. Yet the great majority of physiologically important cellular processes occur in the cytosol or in the nucleus, which is topologically connected to the cytosol by the nuclear pores.

Several intracellular delivery strategies have been developed based on naturally occurring translocation domains or other physical principles. However, not all of them have been scrutinized regarding the fraction of the cargo that actually reaches the cytosol. Most of the delivery strategies described in this review rely on the active uptake of cargo molecules by the cell via endocytosis. Mammalian cells are capable of a number of different endocytosis mechanisms, and their engagement depends on the molecule that is taken up.<sup>1,2</sup> Larger particles and volumes of the extracellular fluid are generally taken up via phagocytosis and macropinocytosis. These two mechanisms operate by the engulfment of extracellular material by the plasma membrane via remodeling of actin filaments and further processing into endosomal compartments. Smaller particles, however, can also be taken up via pathways that depend on coat proteins, specifically clathrin or caveolin. Upon initiation of

endocytosis, clathrin or caveolin coat the bending membrane until the particles are fully engulfed, while further factors, such as dynamin, separate the vesicle from the membrane. Upon uncoating, endocytosed vesicles are processed into early endosomes, and the particles inside are further sorted: for degradation into lysosomes or else into other cellular compartments such as the *trans*-Golgi via retrograde transport. Clathrin- and caveolin-independent pathways have also been described, which can be also dependent or independent of dynamin.<sup>1,2</sup>

One major issue intrinsic to internalization via endocytosis is the entrapment of cargoes within endosomes. Another issue is that upon acidification of these endosomes and further fusion with lysosomal compartments, trapped molecules are degraded.<sup>3</sup> For molecules with cytosolic targets, however, biological activity requires the molecule not just to be taken up by the cell, but to efficiently escape the endosome and access the cytosol. Accessing the cytosol can be accomplished by different mechanisms, which are further detailed in subsequent sections. Some molecules have the intrinsic property of forming pores or using existing protein pores and can thus enter the cytosol from endolysosomal compartments without any impact on the endosome itself. A very different “escape mechanism” relies on the rupture of endosomal membranes. Some delivery systems, as detailed below, can effectively buffer the acidification of the endosome. The high water and ion influx then leads to the rupture of endosomal membranes, allowing the diffusion of the molecule-of-interest into the cytosol.

A critical question for research on cell-penetrating biomolecules is therefore to distinguish with confidence molecules that have been taken up by the cell (*total cellular uptake*) from molecules that have reached the cytosol (*cytosolic localization*). These are very different measurements of “intracellular” localization or “cell penetration”, and we avoid these latter terms because of their imprecise nature. Measuring *total cellular uptake* refers to all material within the cell, including endosomal and lysosomal compartments and, sometimes, material adhered to the plasma membrane. Measuring *cytosolic localization* refers only to material in the cytosol, which is typically the material that will confer a biological effect. Distinguishing between total cellular uptake and cytosolic localization is therefore extremely important in the screening and development of biologically active macromolecules. Recent work has highlighted the concept of translocation efficiency, which can be measured as the ratio of cytosolic localization to total cellular uptake. If both total cellular uptake and cytosolic localization of a cargo are measured, these quantities provide not only a contrast between these two measurements, but a quantitative measure of endosomal escape efficiency for a specific molecule and its translocation mechanism.

In this review, we first describe various translocation strategies with a focus on their endosomal escape mechanisms. We then discuss several assays currently used to measure “cell penetration” in detail and discuss their ability to differentiate between total cellular uptake and cytosolic localization. Finally, we also review some procedures and their associated artifacts that have led to false interpretations of delivered cargo molecules in the past, and thus should no longer be used.

## DELIVERY METHODS AND THEIR MODES OF ACTION

Over the past few decades several different protein translocation methods have been developed. In the next paragraphs we describe the most commonly used delivery methods, which are also summarized in Figure 1.

### Physical Methods.

Some of the oldest and most direct methods to facilitate intracellular delivery are mechanical methods, in which the membrane is physically disrupted during incubation of the cargo molecule. Various physical methods such as electroporation, microinjection, sonoporation, or mechanical deformation were recently reviewed quite extensively.<sup>4</sup> Physical methods for intracellular delivery of macromolecules bypass endocytosis and allow for direct access of exogenous molecules into the cytosol, but they can be highly damaging. Also, most are poorly translatable to live organisms.

### Cell-Penetrating Peptides (CPPs).

Cell-penetrating peptides (CPPs), first developed about 30 years ago,<sup>5</sup> have drawn a great deal of attention due to their potential for nucleic acid and protein delivery.<sup>6–8</sup> Tat and penetratin were the first CPPs to be reported, derived from the natural proteins HIV Tat (“trans-activator of transcription”) and *Antennapedia* homeoprotein, respectively.<sup>9–12</sup> Many synthetic CPPs have since been designed, mostly linear peptide sequences up to 40 amino acids long. CPPs have received this name by their observed property to “penetrate” membranes and translocate themselves and an attached cargo to the cytosol of cells, yet their efficacy and mechanism depend on many factors and are often not clear.<sup>3</sup> Positively charged functional groups, particularly guanidinium groups, are essential for total cellular uptake for most CPPs, and this property is also related to cytosolic localization.<sup>13–23</sup> However, amphipathic, hydrophobic, or even anionic CPPs have also been described.<sup>24,25</sup>

Although there has been quite an effort to clarify the mechanism(s) by which CPPs translocate to the inside of a cell, it remains elusive how exactly a particular peptide or peptide–cargo complex crosses the membrane.<sup>3</sup> One proposed mechanism is the interaction between the positively charged peptide and negatively charged cell surface proteoglycans. Thus, the CPP electrostatically interacts with the membrane itself or its glycoprotein components and gets internalized via endocytosis, without a clearly defined mechanism for a subsequent endosomal escape.<sup>3</sup> Besides rupture of the endosomal membrane (see below), other proposed mechanisms for CPPs are direct penetration, pore formation, micelle formation, membrane thinning, receptor-specific uptake, transporter-mediated uptake, as described previously.<sup>2–37</sup> Various mechanistic investigations such as ATP depletion or low-temperature incubation have been conducted, with contradictory results regarding whether a separate uptake mechanism is relevant other than endocytosis.<sup>18</sup> For instance, it has been shown that direct translocation occurs only upon a certain threshold concentration, but it remains elusive how these high concentrations may affect membrane integrity of cellular and endosomal membranes, as we will discuss below.<sup>38,39</sup> Data also suggest that, at least in some cases, uptake is dependent on the peptide-to-cell ratio rather than on the peptide concentration.<sup>40</sup>

Upon endocytosis, one major problem of CPP-mediated delivery is endosomal entrapment.<sup>41–44</sup> It is generally unclear whether escape from endosomes relies on a specific mechanism, and it likely depends on the physicochemical characteristics of the CPP and attached cargo, its concentration, and/or the cell line and culture conditions used for the assay.<sup>45</sup> Because of the diverse properties of CPPs, a universal uptake mechanism that applies to all molecules will be unlikely.<sup>22,46</sup> However, a few key studies have provided a simple framework for understanding the critical parameters for possible cell penetration mechanisms. Among others Fuchs et al.<sup>34</sup> demonstrated in 2004 that leakage from endocytic vesicles can occur, dependent on high concentrations of CPP.<sup>34,35</sup> In this mechanism, the CPP induces internalization via endocytosis and then leads to rupture of the endosomal membrane, finally “penetrating” into the cytosol.<sup>36,37</sup> We discuss below the potential role of massive hydration and subsequent rupture of the endosomes. These high concentrations thus might lead to toxic effects that need to be tested for each peptide cargo complex.<sup>15</sup>

Since cytosolic localization is not as easily achieved as initially assumed, various efforts have been undertaken to improve delivery efficiencies of CPPs. More recently, machine-learning based predictions of cellular uptake as well as the simulation of improved cargo delivery have been explored to reduce screening efforts for suitable peptide–cargo combinations.<sup>16,23,47–50</sup> Inherent in these efforts is the assumption that the predicted molecular structure can actually enhance endosomal escape, beyond the hydration and rupture effect. It would be extremely crucial to train such algorithms by reliable data on how much cargo has reached the cytosol, compared to how much has remained in endosomes. The importance of distinguishing these data emphasizes the need for a careful approach to data interpretation that is the focus of the present review. Enhancing endosomal escape and therefore increasing cytosolic concentrations are challenges that remain under investigation.  
51

As will be discussed further below, CPPs are part of a larger group of “cell-penetrating” cationic molecules, including supercharged proteins, naturally occurring cationic proteins (such as most DNA-binding proteins), cationic nanoparticles, cationic detergents, and auxiliary substances such as chitosan and polyethylenimine.

### **Zinc Fingers and Other DNA-Binding Proteins.**

Zinc-finger domains and other DNA-binding proteins usually have a high net-positive charge as their natural role is to bind negatively charged DNA. Zinc-finger proteins have up to 20 positive charges for a typical protein with six zinc-finger domains. Similarly to CPPs, their positive charges interact with the cell surface. It is therefore not unreasonable to treat them in one group together with CPPs and supercharged proteins (see next section), and positively charged nanoparticles.

Genome engineering applications have utilized zinc-finger domains by taking advantage of their reported self-delivering properties.<sup>52</sup> Recently, the delivery of proteins and enzymes as cargoes fused to zinc-finger domains has also been reported.<sup>53</sup> Proteins genetically fused to zinc-finger domains appear to enter mammalian cells via macropinocytosis and, to a limited extent, through caveolin-dependent endocytosis.<sup>53</sup> This process is dose-dependent, and delivery efficiencies varied with the number of zinc-finger domains, with an optimum of two

or three domains. This limit appeared to be due to overall protein stability.<sup>53</sup> It is not clear how proteins escape the endosome upon macropinocytosis, but the endosomal escape efficiency varied from 12–80%, depending on the assay used.<sup>53,54</sup> Further studies are clearly needed to elucidate their detailed translocation mechanisms and assess their potential as transporters.

### Supercharged Proteins.

In 2010 another class of protein-based delivery vehicle was described that maximizes the most common feature of successful CPPs and DNA-binding proteins: high positive charge. “Supercharged” GFP containing varying numbers of surface-exposed charges has been reported to potently and rapidly deliver proteins *in vitro* and *in vivo*.<sup>55–57</sup> It was also employed as an effective method to enhance siRNA and DNA transfection.<sup>58</sup> Other naturally occurring human proteins with high positive net charges have also been tested for their delivery potential.<sup>59</sup>

Supercharged proteins seem to utilize uptake mechanisms similar to those of CPPs. This may not be surprising, since the more efficient transfection agents typically have a high positive net charge as well as a minimum proportion of hydrophobic surface area. The positively charged proteins do not passively translocate through the membrane, but rather are endocytosed upon binding to sulfated proteoglycans on the cell surface. This induces endocytosis pathways dependent on clathrin and dynamin, leading to uptake into endosomes. Translocation efficiency is strongly dependent on the net positive charge and can be designed to be pH dependent.<sup>60,61</sup> Supercharged proteins can also have higher net charges than short CPPs, but when molecules of similar net charge are compared, supercharged proteins show higher total cellular uptake. The mechanism of this enhanced uptake remains elusive.<sup>59</sup> It has been proposed that proteoglycan cross-linking is the major driver for macropinocytosis and that the larger surface area is advantageous over CPP-mediated endocytosis.<sup>60</sup> Resistance to proteolysis may also contribute to this effect.

It has been observed that uptake of supercharged GFP is characterized by punctate endosomal staining in peripheral parts of a cell. Some authors have proposed that these endosomal compartments potentially fail to undergo further transport or processing into lysosomal compartments, thus giving more time for endosomal escape of trapped cargo.<sup>60</sup> This hypothesis would view endosomal escape as a competing pathway with lysosomal degradation, with the role of endosomal rupture to be established.<sup>36,37</sup> Clearly, further studies are required to elucidate the exact mechanism of endosomal escape of supercharged proteins.

### Nanoparticles and Liposomes.

Nanoparticles represent a diverse group of carriers designed to deliver small molecules, nucleic acids, peptides, and proteins to the cytosol. Nanocarriers have been used to deliver several proteins, most commonly GFP but also other proteins like CRISPR/Cas9 designed for future therapeutic applications, as reviewed by Yu et al.<sup>62–65</sup>

Various synthetic and naturally occurring polymers, lipids, polysaccharides, and proteins have been used as carriers for nanoparticle-based delivery.<sup>66–70</sup> Many parameters must be

considered when designing nanoparticles to encapsulate, deliver, and release cargo, including size, aggregation rate, surface characteristics, efficiency of encapsulation, and drug release kinetics.<sup>66,67</sup> The most common drawbacks of these carriers are their high cytotoxicity, low stability, variable efficacy, and various issues associated with biocompatibility and biodegradability. These drawbacks vary widely among different nanocarriers and thus must be tested carefully for each individual carrier and cargo.<sup>67,71,72</sup> The most prominent synthetic polymers used as nanocarriers for protein delivery are copolymers consisting of polylactic-*co*-glycolic acid (PLGA), *N*-(2-hydroxypropyl) methacrylamide, or polyvinylpyrrolidone.<sup>70</sup> Several proteins, such as albumin, have been cross-linked to form protein-based nanoparticles. These carriers represent promising alternatives to synthetic nanoparticles due to reduced toxicity and reduced affinity to off-target tissues.<sup>66,67,72</sup>

The proposed uptake mechanism for nonliposomal nanocarriers depends on the nature of the carrier. Interaction with the cellular membrane leads to, among others, different types of endocytosis, phagocytosis, macropinocytosis, or direct membrane fusion. Several mechanisms have been proposed for endosomal escape; however, various nanocarrier–protein complexes have been shown to remain trapped in the endosome and undergo lysosomal degradation, leading to low cytosolic levels of cargo molecules.<sup>72</sup>

As with other delivery methods, endosomal escape of nanoparticles is a complicated and diverse process that can involve several different mechanisms.<sup>73,74</sup> Some cytosolic delivery methods take advantage of the “proton sponge” effect to mediate endosomal escape. Membrane pumps create a proton gradient within the late endosome, leading to acidification, a process stabilized by a carrier with buffering capabilities. Negatively charged ions and water molecules flood into the endosome from the cytosol to offset the proton gradient. This high influx of ions results in endosomal rupture and release of trapped cargo. Cationic polymers often have considerable pH-buffering capacity, enhancing endosomal escape through the same mechanism. However, as previously mentioned for CPP-mediated delivery, high net positive charge may lead to cellular toxicity. To decrease overall toxicity, pH-sensitive nanocarriers have been designed, such as carriers based on calcium phosphate. They have a neutral charge in circulation and reveal their buffering or membrane disruption capabilities only in the acidified endosome.<sup>74</sup> Nanocarriers that are membrane-disruptive are believed to have a high endosomal escape efficiency.<sup>74</sup>

Nanoparticles based on liposomes can be treated as a separate class of carriers as they can effectively fuse with the cellular or endosomal membrane, thereby delivering their encapsulated cargo to the cytosolic space. Liposomes with CPPs on their surface have further shown enhanced cellular uptake and endosomal escape.<sup>75–77</sup>

Additional nanocarrier-based delivery systems have been developed with different proposed mechanisms. Hydrophobic polyampholytes have been proposed to interact with and destabilize the endosomal membranes leading to cargo release to the cytosol.<sup>72</sup> Another method, a freeze concentration protocol, was able to result in highly internalized complexes; however, a clear understanding of the uptake mechanism and endosomal escape remains elusive, and this particular method is only suitable for *in vitro* delivery.<sup>71</sup>

### Virus-like Particles.

Compared to other delivery methods, virus-like particles (VLPs) are still in their infancy. VLPs have been generated both from enveloped and nonenveloped viruses. The nonenveloped ones are tightly packed and ordered assemblies of viral coat proteins, but they are devoid of viral DNA or RNA. Because of these characteristics, VLPs have been used extensively as vaccine carriers, since the high immunogenicity of these particles is ideal for vaccine applications.<sup>78–80</sup> VLPs from many different virus types have been used as protein delivery platforms.<sup>81–84</sup> Cargo proteins are either fused to the surface of the VLP or encapsulated within the VLP during their self-assembly. Through attaching receptor-targeting molecules to surface proteins, VLPs can also be made cell-specific.<sup>81</sup>

Although natural viruses possess the ability to transduce cells and deliver nucleic acids to the cytosol, usually with subsequent transport to the nucleus, the exact mechanism by which a cargo molecule reaches the cytosol from a VLP remains elusive. There are some data to support a model in which, upon receptor-mediated endocytosis, the VLPs escape the endosomes by their natural endosomal escape mechanism. This function would have to be provided by one of the viral coat proteins.<sup>85</sup>

### Bacterial Toxins.

Some bacteria naturally secrete protein toxins with a component that can translocate a toxic protein to the cytosol of host cells. These toxins have evolved a highly specific translocation mechanism to deliver to the cytosol an enzyme that fatally damages the cell. The best studied bacterial translocation complexes are those associated with anthrax toxin (*Bacillus anthracis*), diphtheria toxin (*Corynebacterium diphtheriae*), and exotoxin A (*Pseudomonas aeruginosa*).<sup>86,87</sup> These bacterial protein toxins are of the AB type and usually consist of three major domains, an A-component with an enzymatic/toxic domain, as well as a B component with a cell-binding domain and a translocation domain.

Because of their modular structure, domains can be engineered for altered cell specificity and customized cargo, making bacterial protein toxin transport systems an adaptable and potentially general approach for protein delivery.<sup>88–92</sup> For instance, more than 30 cargo proteins have been tested in anthrax-mediated delivery, as reviewed previously.<sup>86</sup> A prerequisite for engineering bacterial toxins is a thorough understanding of the natural translocation mechanisms, which are not yet fully understood and have also been reviewed elsewhere.<sup>87,93</sup> Here we provide a brief overview of these mechanisms, specifically focusing on AB toxins that have been engineered with altered cell specificity and alternative cargoes.

For effective, cell-specific targeting, the natural binding domain of AB toxins must either be completely replaced by a retargeting molecule,<sup>94</sup> or mutated to prevent wild-type receptor binding and additionally fused to a binding molecule for cell-specific interaction.<sup>95</sup> Since the receptor-binding domains are loosely associated with the translocation domain, they can be exchanged in a modular fashion. Successful retargeting of various toxins has been shown for tumor-associated receptors like EpCAM and Her2.<sup>96–98</sup> Upon receptor binding, proteolytic activation of some toxins occurs at the cell surface. Wild-type protective antigen (PA), the translocation domain of anthrax toxin, is proteolytically activated by furin; however, this



cleavage site can be altered to a substrate for proteases that are highly overexpressed in various cancer types.<sup>99,100</sup> The toxin complexes get internalized via receptor-mediated endocytosis. For a number of toxins, it is not yet fully understood if this process is clathrin-dependent or -independent.<sup>101</sup> In the early endosomes, proteolytic activation of some toxins occurs at this stage by cleaving off the A component.<sup>87</sup>

Once inside endosomes, further processing differs depending on the toxin. Two distinct endosomal escape mechanisms have been described that ensure cytosolic delivery and prevent endosomal degradation. The first, used by Shiga toxin and *Pseudomonas* exotoxin A (ETA), relies on the cellular translocation machinery via the *trans*-Golgi network and the endoplasmic reticulum (ER). Shiga toxin traffics through the Golgi apparatus directly from early endosomes,<sup>101</sup> whereas ETA requires a pH shift as occurs in late endosomes before undergoing a similar retrograde transport. Both translocate their cargo via the Golgi to the ER and eventually to the cytosol. Cargoes that undergo retrograde transport rely on the unfolding and refolding machinery of the host cell, and it is unclear how efficiently alternative cargoes get processed. The rate-limiting step, however, is the efficiency by which proteins get translocated from the endosome to the *trans*-Golgi network, with a proposed efficiency of 5–10%.<sup>93</sup>

The other endosomal escape mechanism, used by anthrax toxin, diphtheria toxin, *Clostridium botulinum* C2 toxin and others, directly translocates cargo proteins across the endosomal membrane. Diphtheria toxin and anthrax toxin might require a pH shift to late endosomes, depending on the receptor to which they are bound.<sup>102</sup> Direct translocation from these late endosomes then occurs by insertion of a pore, formed by a domain of its B component, into the endosomal membrane. Because of the lower pH of the late endosome, the bound cargo molecules unfold and translocate to the cytosol through this pore.<sup>90,103</sup> Direct translocation via toxin-encoded membrane pores is independent of any cellular machinery but requires the cargo to have a certain surface charge to pass through the selective pore and the ability to unfold prior to translocation and to refold after translocation. The delivery of alternative cargoes with bacterial protein toxins was observed to have a wide range of efficiencies, depending on the energy needed to unfold the protein.<sup>96</sup> It has been shown that the native toxins must refold to become catalytically active and effectively kill the host cells,<sup>104</sup> and this also applies for alternative cargoes.

## ASSAYS FOR TOTAL CELLULAR UPTAKE

From CPPs to supercharged proteins to VLPs, a key aspect of research into cell-penetrating molecules is the assay used to measure cellular internalization. As defined above, it is prudent to distinguish clearly between assays that measure *total cellular uptake*, which may include endosomally trapped material, and assays that specifically measure *cytosolic localization*. In this section and in Table 1, we describe the development of assays that measure total cellular uptake. Notably, while measurements of total cellular uptake remain valuable (for instance, in calculating endosomal release efficiency), we emphasize that *these assays do not measure cytosolic localization*. Assays that do measure cytosolic localization are described in the next section. The results of assays that measure total cellular uptake and those assays that measure cytosolic localization are two different measurements, and these

do not necessarily correlate with each other. Early on, these two measurements were routinely conflated, but recent evidence has demonstrated the critical importance of distinguishing them because, in general, only cytosolically present molecules can elicit a biologic effect (see section below entitled Artifacts and Misinterpretations in Measurements of Total Cellular Uptake and Cytosolic Localization).

### **Assays That Measure Passive Translocation Across Membranes and Cell Monolayers.**

For decades, medicinal chemists have been developing and refining assays for predicting oral bioavailability, typically of small molecules. These assays do not measure cytosolic localization directly, but their results have generally correlated well with cytosolic localization of typical small-molecule drugs.<sup>105-107</sup> Some assays commonly used in medicinal chemistry employ synthetic model membranes, while others use cell monolayers. Despite being developed to predict gut absorption of small molecules, these assays have also been used to characterize biological macromolecules, especially to identify those which may enter cells via a passive penetration mechanism.

**Synthetic Model Membranes.**—Before 1998, measuring translocation across synthetic model membranes typically involved chromatography with immobilized artificial membrane (IAM) columns.<sup>108</sup> To address the low-throughput nature of IAM chromatography, Kansy et al.<sup>109</sup> developed the parallel artificial membrane permeability assay (PAMPA). PAMPA measures diffusion of a molecule-of-interest across an artificial lipid bilayer. Originally, UV spectroscopy was used to measure the flux of molecules across the artificial membrane,<sup>109</sup> but modern implementations commonly use quantitative LC-MS.<sup>107,110</sup> Variations of PAMPA have recently been reviewed elsewhere.<sup>111</sup> As an alternative to PAMPA, a few groups reported similar assays for passive penetration of synthetic membrane vesicles.<sup>112,113</sup> For instance, synthetic vesicles were used to monitor the internalization of dye-labeled molecules-of-interest by quantitating the flux across the membrane by HPLC.<sup>112,113</sup> Similar work was performed with synthetic giant unilamellar vesicles that have enclosed inner vesicles containing a membrane-impermeant fluorescent dye.<sup>114</sup> This assay measures membrane permeabilization because the giant vesicle will only become homogeneously fluorescent if a peptide transiently permeabilizes the membrane.<sup>114</sup> Synthetic multilamellar vesicles can also measure the extent of membrane translocation of a dye-labeled cargo by fluorescence microscopy.<sup>115</sup> The same setup, but with a fluorescence polarization readout, was used to determine if fluorescein-labeled cyclic peptides bound extensively to membrane phospholipids of small unilamellar vesicles.<sup>116</sup>

Artificial membrane assays are excellent assays to measure the behavior of molecules with respect to the phospholipid bilayer. When it comes to being proxies for cell penetration of live cells, there are several important limitations. These assays measure only passive penetration of the lipid composition studied and cannot take into account any effects due to protein binding outside or inside the cell, any effects of cellular internalization pathways such as endocytosis, and most importantly, endosomal escape mechanisms. Despite this caveat, the results obtained from these types of assays have proven to correlate well with cytosolic localization mostly for small molecules, but also for a subset of CPPs, some intrinsically cell-penetrant peptides, and some CPP-cargo complexes.<sup>107,111,117,118</sup> Since the

assay by itself does not measure cytosolic localization, any hypothesis derived from these assays needs to be verified by true cytosolic localization assays (section below entitled Assays that Quantitate Cytosolic Localization).

**Monolayer Assays.**—Two assays are commonly used in medicinal chemistry to detect translocation across a cell monolayer. The first uses human colon adenocarcinoma (Caco-2) cells, which spontaneously grow in monolayers and differentiate into polarized enterocytes, and therefore mimic many of the properties of enterocytes in the small intestine.<sup>119,120</sup> Caco-2 monolayers are grown on a transwell permeable support to allow quantitation of the translocation of molecules-of-interest across the monolayer.<sup>121,122</sup> Originally, a liquid scintillation counter was used to monitor the translocation of radioactive molecules that had reached the basal side of the Caco-2 monolayer,<sup>122</sup> but LC-MS is the current detection method of choice.<sup>123,124</sup> The second assay, inspired by the Caco-2 monolayer system, uses Madin-Darby canine kidney (MDCK) cells.<sup>125</sup> MDCK cells proliferate more quickly than Caco-2 cells and have more stable expression of membrane transporters, making them an attractive alternative, but both assays are still commonly used.<sup>126,127</sup>

Caco-2 and MDCK assays are commonly used as a proxy measure for small-molecule cell permeability with the goal of predicting oral bioavailability. Cell monolayer assays can be high-throughput, and their results typically correlate well with gut permeation in vivo.<sup>120,128</sup> However, it is important to note that they monitor both transcellular transport (which depends on total cellular uptake and, potentially, cytosolic localization and the release on the distal side) and paracellular transport (in which cargo passes through junctions between cells and does not enter these cells).<sup>120,122,129</sup> Paracellular transport would not be expected to be related to cytosolic localization, and transcellular transport of macromolecules may be vesicular, meaning that the traversing molecule may never enter the cytosol.

Overall, synthetic model membranes remain a useful tool for predicting potential cytosolic localization for molecules-of-interest, but only for a subset of molecules expected to enter cells passively. Yet, neither total cellular uptake nor cytosolic localization of a molecule can be measured directly using these assays.

### **Assays That Measure the Amount of Molecule in a Cell Lysate.**

One approach for measuring total cellular uptake is to quantitate the amount of molecule-of-interest in a cell lysate, as opposed to detecting molecules in intact cells. The general approach for these cell lysate assays is to incubate cells in culture with a molecule-of-interest, lyse the cells, and quantitate the amount of molecule present within the cell lysate (typically analyzing only the soluble fraction of the lysate). Quantitation can be accomplished in several ways, most notably HPLC, mass spectrometry, peptide nucleic acid (PNA) hybridization, and quantitative Western blotting (Figure 2). Typically, stringent washing is necessary to try to remove material from the exterior of the cell prior to lysis, in order to quantify only internalized material, and it is not always easy to verify that all external material was truly removed.

**Shift in HPLC Retention Time after Chemical Labeling.**—One of the earliest cell penetration assays relied on chemically labeling extracellular and surface-bound molecules,

in a manner that did not label internalized molecules. HPLC was then used to separate the unmodified form of the molecule-of-interest to detect total cellular uptake. For this purpose, Oehlke et al.<sup>129</sup> incubated cells in culture with a nonlabeled peptide of interest and then washed cells and treated with a diazotized 2-nitroaniline, which reacted with extracellular and surface-exposed peptide. The cell lysates were then analyzed by reverse-phase HPLC. Material that eluted at the normal retention time was interpreted as having been internalized by the cells, while modified peptides showed a shift in retention time; the relative amounts of modified and unmodified peptide were used to make conclusions on the degree of internalization.<sup>129,130</sup>

**Mass Spectrometry.**—Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) has most commonly been used as a label-free method to quantitate the total internalization and degradation of peptides in cell lysates and conditioned cell culture media. To ensure reliable quantitation, a deuterated form of the molecule-of-interest was used as an internal calibration standard.<sup>131–133</sup> To reduce the background signal from the lysate, Chassaing and co-workers<sup>134,135</sup> used a biotinylated molecule to enable a preanalysis purification step. Specifically, cultured cells were incubated with a biotin-labeled molecule, washed, and lysed, and then the lysate was spiked with a known concentration of deuterated, biotinylated molecule as an internal standard. Biotinylated molecules were purified with streptavidin-coated magnetic beads, and the mass spectrometry peak ratio of nondeuterated to deuterated molecules allowed for the quantification of the internalized molecule-of-interest.<sup>134–137</sup> More recently, a label-free variation was developed that used size-exclusion chromatography as a purification step, followed by reverse-phase HPLC.<sup>138</sup>

Mass spectrometry is very sensitive, allowing the measurement of small amounts of internalized cargo. One drawback to mass spectrometry-based detection methods is the need for an internal standard for accurate quantitation. Most commonly, a deuterated form of each molecule must be prepared as the standard, which places limits on throughput with respect to the number of different molecules that can be analyzed in parallel.

**PNA Hybridization Assays.**—Peptide nucleic acid (PNA) hybridization assays are used to quantitate the amount of nucleic acid (often, therapeutic RNA) in a sample. PNA hybridization has been used routinely to monitor total cellular uptake of nucleic acids for cells in culture and accumulation in tissues *in vivo*. To detect an RNA of interest, cell lysates are incubated with a complementary, dye-labeled PNA either in solution or with the PNA immobilized as a capture probe. The RNA that is captured through hybridization to the PNA is subsequently quantitated by HPLC.<sup>139,140</sup>

This detection method is highly specific, and it also allows for detection of internalization in *ex vivo* tissue samples just as easily as in cell culture.

**Quantitative Western Blotting.**—While less common, quantitative Western blotting can also be used to measure the amount of a cargo in a cell lysate. Quantitation is achieved by normalizing band intensities to those of loading controls of known concentration.<sup>141</sup> This method is limited to the analysis of molecules that have an epitope for a detection antibody, such as proteins or peptides carrying an affinity tag.

## Assays That Measure the Amount of Molecule in Intact Cells.

Measuring the amount of cargo in a cell lysate has the advantage of producing a bulk signal for a large number of cells. This can lead to increased sensitivity, especially for molecules that have small degrees of total cellular uptake. By contrast, many assays have been developed that measure internalization of the molecule-of-interest at the level of individual, intact cells. These assays can have decreased sensitivity, but allow for more direct measurement of total cellular uptake. In this section, we discuss assays that measure total cellular uptake in intact cells without quantitative measurement of cytosolic localization. These assays integrate signal from material localized in any compartment, including those trapped in endosomes and lysosomes. Just as for assays using cell lysates, these assays require efficient removal or exclusion from the assay of molecules still bound to the surface.

**Fluorescence-Based Methods.**—The most common methods used to monitor the cell penetration of biomolecules involve conjugating a fluorescent dye to the molecule-of-interest, then observing or quantitating the localization of the dye. The extent of internalization is most often measured by fluorescence microscopy or flow cytometry.

Fluorescence microscopy is only qualitative with respect to the amount of fluorophore delivered and the relative localization of the fluorescence throughout the cell. A common phenomenon observed is punctate staining due to endosomal entrapment. However, if the microscope cannot resolve individual puncta, and/or if it cannot eliminate out-of-plane fluorescence, then diffuse fluorescence can be erroneously interpreted as cytosolic localization. Even confocal fluorescence microscopy can have difficulties distinguishing dye-labeled molecule in the cytosol from small aggregates, or molecules that are trapped inside small endosomes or lysosomes.<sup>24,142–144</sup> Careful high-content microscopy, custom-tailored algorithms and colocalization with lysosomal/endosomal markers can increase the reliability and reproducibility of analyzing images from fluorescence microscopy, but not necessarily the quantitation and definitive discernment of cytosolic material.<sup>145</sup> Fluorescence microscopy, even confocal microscopy, is thus very challenging for quantitating total cellular uptake and can be unreliable for determining cytosolic localization.

An appealing alternative for measuring the degree of association of dye-labeled cargo with cultured cells is flow cytometry. Flow cytometry is quantitative and very high-throughput. However, flow cytometry cannot provide even qualitative data on the subcellular localization of a dye-labeled cargo. Thus, it cannot distinguish endosomally trapped material from cytosolic material. Furthermore, signals can include fluorescence from dye-labeled molecules associated with the cell membrane.<sup>18</sup> To address this issue, washing steps with an enzymatic digest or trypan blue have been used to reduce or somewhat quench fluorescence of dye-labeled peptides at the outer membrane.<sup>146</sup> Additionally, trypsin and/or heparin treatment can be used to eliminate excess signal from noninternalized molecules at the cell membrane, though the completeness of this step must be verified.<sup>146,147</sup>

Often, a combination of fluorescence microscopy and flow cytometry has been used for qualitative assessment of subcellular localization and quantitative measurement of total cellular uptake, respectively. This approach can distinguish highly penetrant molecules from

poorly penetrant molecules, but may not be suited for careful comparisons among subtly different molecules. Another limitation is that all fluorescence-based assays require a bulky, hydrophobic dye to be attached to the molecule-of-interest. As with all chemical tags, attachment of a fluorescent dye can alter the solubility, cellular uptake, and endosomal escape of the tagged molecule.<sup>148–150</sup> If the molecule-of-interest can be degraded in the culture medium or in the cell (particularly relevant to lysosomally trapped material), the dye may become cleaved from the cargo, leading to staining of the cytosol and misinterpretation of the results. One approach that could sidestep some of these drawbacks is to label the molecule in situ after incubation of the molecule-of-interest with the cells, but high background and low sensitivity have prevented this approach from being widely adopted.<sup>151</sup>

Overall, only carefully interpreted data from microscopy and flow cytometry have generally correlated with results from mass spectrometry and other cell penetration assays that measure total cellular uptake,<sup>146,152,153</sup> but none of these methods are suitable for measuring cytosolic localization.

**Ion Conductance Microscopy and Electrochemical Microscopy.**—In 2017, Unwin and co-workers<sup>154</sup> used scanning ion conductance microscopy (SICM) and scanning electrochemical microscopy (SECM) in tandem to monitor the internalization of biomolecules across specific, localized patches of the cell membrane. This assay involved a two-barrel probe system in proximity to a single cell. One compartment of the probe contained the molecule-of-interest, and the other housed a carbon electrode that measured the local concentration and flux of molecules as they were delivered across the plasma membrane.<sup>154</sup> This method measures not only the amount of uptake, but also the rate of uptake, at several localized areas of a single cell. Yet, the method cannot distinguish the subcellular localization of the molecule-of-interest.

## ASSAYS THAT QUANTITATE CYTOSOLIC LOCALIZATION

Methods that monitor the total cellular uptake of biomolecules were at one time widely accepted and trusted as measurements of “cell penetration”. It was common 10 years ago to assume that these measurements were reflective of a molecule’s cytosolic localization. As the fields of peptide, protein, and nucleic acid delivery grew, increasing evidence surfaced that common methodology was not adequate, as it did not report on cytosolic localization, but rather total cellular uptake, which includes membrane-associated and endosomally trapped material.

While these assays were standard several years ago and still offer useful information on total cellular uptake, most investigators today are careful to avoid conflating these two measurements. As described in this section and in Table 2, several methods have been developed to measure the degree to which a molecule-of-interest reaches the cytosol, distinct from total cellular uptake. As a whole, cytosolic localization assays have largely surpassed total internalization assays as the methods of choice for detecting cellular penetration.

### Assays That Separate the Cytosol Using Ultracentrifugation.

As mentioned above, one of the major drawbacks of analyzing cell lysates is that the lysate is a mixture of all cell-associated material, including material from the cytosol, organelles, vesicles, and plasma membrane. To isolate the cytosolic fraction from membrane-enclosed compartments, a lysate can be physically separated by high-speed ultracentrifugation. Subcellular fractionation is a common technique in cell biology, where Western blotting is used to confirm fractionation of different compartments and to compare the levels of endogenous biomolecules in those compartments.<sup>156–158</sup> Applied to any of the lysate-based cell penetration assays described above, fractionation potentially allows independent quantitation of the amount of molecule-of-interest localized in each cellular compartment. However, subcellular fractionation is technically demanding, particularly for separation of small endosomes from cytosolic material. It can also be difficult to verify complete separation of each compartment, particularly if the cargo is small enough to diffuse through membranes permeabilized in the lysis or fractionation process.<sup>159</sup>

### Assays That Distinguish Cytosolic Fluorescence.

Some of the first assays designed to selectively measure the cytosolic access of a molecule-of-interest exploited the unique chemical environment of the cytosol. In 2001, Langel and co-workers<sup>160</sup> labeled molecules-of-interest with a fluorescence quencher and further conjugated these to a fluorophore-containing peptide via a disulfide bond. Treatment of cultured cells with these disulfide-conjugated, self-quenched molecules would result in a fluorescence signal only if the disulfide was broken, which was assumed to only happen in the reducing environment of the cytosol (Figure 3a). However, one drawback of this assay is the possibility of disulfide reduction in the endosome or even at the cell membrane, making it harder to distinguish between different cellular compartments.<sup>161,162</sup> This approach required a large label, but it did allow monitoring of the kinetics of accumulation, albeit not the subcellular localization.

Another fluorescence-based method takes advantage of the pH difference between the cytosol and the endolysosomal compartment by conjugating a pH-sensitive dye, naphthofluorescein, to the molecule-of-interest (Figure 3b).<sup>163</sup> Naphthofluorescein is nearly completely protonated and exhibits no fluorescence when trapped in the endosome or lysosome, where the pH is between 5 and 6, but becomes fluorescent when it escapes the endosome and enters the cytosol.<sup>163,164</sup> Naphthofluorescein fluorescence thus reports on any cellular compartments with neutral pH, so careful additional analysis is needed to distinguish between cytosolic staining and other neutral, membrane-enclosed compartments. Notably, by comparing the ratio of fluorescence from internalized rhodamine-labeled molecules to that of internalized naphthofluorescein-labeled molecules, the relative efficiencies of endosomal escape for different molecules-of-interest could be estimated.<sup>35,163,165–167</sup>

While these assays were designed to report exclusively on cytosolic localization, they required additional independent information, and they lacked absolute quantitation. Recently, a new fluorescence-based method was reported that provided absolute quantitation of fluorophore concentration in the cytosol (Figure 3c).<sup>146,168,169</sup> This method uses

fluorescence correlation spectroscopy (FCS) to measure the diffusion of a dye within a small, defined focal volume of the cell over time, as calculated using a modified three-dimensional diffusion model.<sup>146,168,169</sup> For FCS measurements to report exclusively on the cytosol, focal volumes must be manually selected to exclude areas likely to contain endosomes or other membranes or compartments. This judgment requires both excellent spatial resolution and familiarity with the morphology of the particular cell line under study. FCS correlated well with related measurements using fluorescence microscopy and flow cytometry, but with the ability to exclude material trapped in endosomes or bound to membranes.<sup>153,168</sup> FCS was later combined with independent FACS measurements to compare both total cellular uptake and cytosolically localized material.<sup>169</sup> A dual-color variation of FCS, fluorescence cross-correlation spectroscopy (FCCS), was developed to study the dynamic colocalization of two molecules labeled with orthogonal fluorophores.<sup>170</sup> Provided that the chosen cytosolic region does not inadvertently contain other compartments, a major advantage of FCS and FCCS is that they provide an absolute measurement of cytosolic concentration, while most other assays provide only a measurement of relative concentration.<sup>168</sup> While FCS relies on manual identification of nonendosomal locations that may be difficult to cross-validate with other methods, the published data correlate well to other measures of cytosolic localization.

There are several important drawbacks for all cell penetration assays that measure localization of a dye-labeled cargo. Foremost among these are the potential effects of the dye itself. The dye changes the physicochemical properties of the molecule-of-interest (or CPP cargo), often in ways that can affect uptake and endosomal escape. Thus, results with a dye-labeled molecule may not be representative of the cytosolic localization of the molecule alone. In addition, if the molecule can be degraded in the culture medium or in the endolysosomal compartment, this can lead to release of the dye and diffusion to cellular compartments which are not accessible to the molecule-of-interest being investigated. These drawbacks are characteristic of any assay that requires covalent labeling of the molecule-of-interest. Ultimately, the potential effects of the dye and of cargo degradation must be estimated and minimized using carefully designed control experiments.

### **Assays that Measure Expression of a Reporter Protein.**

Several research groups have produced cell lines that report on cytosolic localization of an exogenously added molecule-of-interest by turning on expression of a reporter protein. An early example of this approach is the luciferase splice-correction assay (Figure 4a), used to monitor the cytosolic localization of a molecule-of-interest labeled with a short, splice-altering RNA.<sup>171,172</sup> The assay used a cell line that incorporated a luciferase gene interrupted by a mutated  $\beta$ -globin intron. Upon delivery to the cytosol, the RNA label spliced out the intron and allowed for the expression of active luciferase, which was quantified using luciferin as a substrate.<sup>171,172</sup>

In 2004, Dowdy and co-workers<sup>173</sup> developed a similar approach using Cre recombinase.<sup>173,174</sup> Cells expressed a reporter gene for eGFP or luciferase, but the reporter gene was disrupted by a loxP site. Reconstitution of the active, expressible reporter gene was achieved by delivery of active Cre to the nucleus. For instance, Tat-Cre fusion proteins were incubated



with mouse T cells in culture, and delivery of Cre was measured by eGFP expression using fluorescence microscopy and flow cytometry.<sup>173</sup> This method reports unambiguously on the delivery of Cre to the nucleus, but only a single turnover of one Cre molecule is needed for regenerating the active gene, after which the signal is amplified by transcription and translation. Thus, while very sensitive, the output of this assay is unlikely to scale linearly with the amount of Cre delivered and may overestimate delivery efficiency for methods with poor endosomal escape efficiency.

In 2005, Kodadek and colleagues<sup>175</sup> reported a different approach to measuring cytosolic delivery using expressed reporters (Figure 4b). This assay used cells transfected with plasmids encoding luciferase downstream from a Gal4 promoter and a hybrid transcription factor made up of Gal4 and the glucocorticoid receptor (GR).<sup>175</sup> Dexamethasone, a GR ligand, was covalently attached to the molecule-of-interest. When the dexamethasone-tagged molecule reached the cytosol, binding of dexamethasone to GR induced translocation of the transcription factor to the nucleus and thus expression of the reporter. An increase in the luciferase signal was correlated to increased extent of cytosolic localization, allowing the comparison of hundreds of peptides and peptoids in a high-throughput format.<sup>175–181</sup> Schepartz and co-workers<sup>54</sup> reported improved versions of this assay that used a modified GR that binds much more specifically to dexamethasone, as well as eGFP instead of luciferase to provide a linear, stoichiometric readout. A direct fusion of GR to eGFP was similarly used to monitor cytosolic localization based on quantitating the degree of nuclear localization of the GR-eGFP conjugate.<sup>54</sup>

Each of these dexamethasone-based assays is high-throughput, sensitive, and cytosol-specific. Because the signal obtained from transcription-dependent assays is amplified, they allow for greater sensitivity for comparing molecules with low levels of cytosolic localization. However, transcriptional amplification also means that the signal is not linearly correlated with the extent of penetration; the assay that monitors the extent of nuclear localization of GR–eGFP relies on a nonamplified signal and may better provide quantitative comparisons among cell-penetrant molecules. Even with these methods, it is difficult to obtain absolute quantitation.

Another method commonly used to measure cell penetration of biomolecules can be grouped as protein complementation assays (Figure 4c), including reporters such as split-GFP,<sup>182</sup> split-luciferase,<sup>183</sup> and split-ubiquitin.<sup>184</sup> GFP can be separated into two fragments, with a smaller peptide containing only 15 amino acids and the larger fragment being nonfluorescent. When combined, the two fragments complement to a functional, fluorescent GFP.<sup>182,185</sup> To assay cytosolic localization, the smaller fragment was conjugated to molecules-of-interest, and the larger fragment was transiently or stably expressed in the cytosol.<sup>186</sup> Once the molecule enters the cytosol, the peptide bound the incomplete, larger GFP fragment, and the resulting fluorescent signal was quantified by flow cytometry.<sup>186</sup> Many variations of this strategy have been reported with different reporters and tags, often investigating both cytosolic localization and disruption of protein–protein interactions in live cells.<sup>51,187–196</sup> These assays, while reporting exclusively on cytosolic access, are still an indirect measure of cytosolic localization because they depend on the extent to which the molecule accesses the cytosol, but also its binding to its unique cellular target.

Tag-based reporter assays share some of the same issues as dye-dependent assays, namely, that the chemical tag may alter cargo properties and that degradation of the cargo in lysosomes may release the tag, leading to artifacts that falsely indicate efficient cytosolic localization. For all reporter assays, a stable cell line would ideally be produced, since throughput and reproducibility can be limited by the need to transfect cells with reporter plasmids prior to each run of the assay, as transfection efficiencies will vary. Finally, as noted above, any signal amplification will increase sensitivity, but will also make the signal nonlinear with respect to the amount of cargo in the cytosol. Overall, these drawbacks are relatively mild, and reporter-based assays continue to see robust use, especially for specific and high-value drug targets for which cell line construction is worth the investment.

### Assays That Measure Direct Interactions with Cytosolic Enzymes.

More recently, several groups have devised assays that rely on cytosolic enzyme activity to generate a quantitative signal. The general principle of these assays is to make the readout dependent on a cytosolically localized enzyme, thus reporting exclusively on cytosolic localization of the cargo. An early example of this strategy was the farnesylation penetration assay (Figure 5a) developed by Falnes et al.<sup>197</sup> The delivered protein was engineered with a C-terminal CaaX motif, a tetrapeptide that is a well-characterized farnesylation substrate. Following addition of the CaaX-labeled protein to the cell, the extent of protein farnesylation was analyzed by SDS-PAGE and correlated to cytosolic localization.<sup>197</sup> The molecule-of-interest is given a membrane association, however, by the farnesyl anchor.

The farnesylation assay inspired a ubiquitin-based assay (Figure 5b), which monitored a protein's access to the cytosol using ubiquitin (Ub) cleavage. After cytosolic delivery of the Ub-protein fusion, ubiquitin-specific C-terminal proteases recognized and cleaved Ub.<sup>198,199</sup> The Ub-protein fusion was fluorescently labeled, so the extent of cleavage could be monitored by SDS-PAGE and Western blot.<sup>198</sup> Notably, using Ub as a tag is prohibitively large for most small molecules, nucleic acids, and peptides, and so this system is most practical for quantitating protein penetration.

Several enzyme-activated fluorogenic probes exist that can assay cytosolic localization (Figure 5c); these have been extensively reviewed previously.<sup>200</sup> One common example requires labeling a cargo with fluorescein-di- $\beta$ -D-galactopyrano-side (FDG, a di-*O*-glycosylated derivative of fluorescein).<sup>201–203</sup> In this assay, the two sugars are cleaved off by  $\beta$ -galactosidase expressed in the cytosol of transfected cells, resulting in the unmasking of fluorescein. The dye is then localized and quantified by fluorescence microscopy and flow cytometry.<sup>201,202,204</sup> A similar system was reported that uses an acetoxymethyl ester of coumarin-cephalosporin-fluorescein (CCF2-AM), which is a FRET-based substrate probe developed specifically for *Escherichia coli* TEM- $\beta$ -lactamase.<sup>203,205–207</sup> CCF2-AM exhibits green fluorescence, but its emission wavelength switches to blue upon cleavage by cytosolic  $\beta$ -lactamase,<sup>208</sup> which was monitored by fluorescence microscopy and flow cytometry.<sup>209,210</sup> The cytosolic calcein release assay' originally developed to measure cell viability' involves either covalently labeling the cargo with calcein-AM, or coencapsulating the cargo with an acetoxymethyl ester version of calcein (calcein-AM) within a liposome to investigate whether the cargo disrupts membrane integrity.<sup>209,211–213</sup> When the

nonfluorescent calcein-AM reaches the cytosol, it is cleaved by cytosolic esterases, releasing fluorescent calcein which can be measured by microscopy or flow cytometry. A similar assay using luminescence instead of fluorescence for more sensitive detection involves conjugation of the molecule to luciferin with a disulfide bond. The release of luciferin from the molecule-of-interest in the reducing environment of the cytosol allows for detection via exogenously expressed luciferase in the cytosol.<sup>214</sup> This approach reports on the cytosolic location of the molecule, with the limitation that any luciferin cleaved upon degradation of the molecule-of-interest would also passively diffuse to the cytosol.

Instead of cleaving a functional group from the labeled molecule; the biotin ligase assay (Figure 5d) introduced by Pluckthun and co-workers<sup>96</sup> relies on biotinylation of the cargo by a cytosolically expressed biotin ligase (*E. coli* BirA).<sup>96,215</sup> This requires fusing the cargo to an avi-tag, which is a 15-residue substrate for BirA<sup>216–218</sup> but not a substrate of eukaryotic biotin ligases. Importantly, the assay can distinguish between total cellular uptake and cytosolic localization. Total internalization can be measured by a second tag fused to the cargo, while cytosolic localization is independently measured by blotting for biotinylated protein.<sup>96,219</sup> This provides a means of calculating the relative efficiency of endosomal escape, as well as the absolute amount of cytosolic and total molecule-of-interest when Western blots are compared to calibration curves with known standards. The Western blots also test for the integrity of the molecule but limit sample throughput.

A conceptually similar assay, but with a smaller tag, was reported in 2017 by Kritzer and co-workers.<sup>220,221</sup> The chloroalkane penetration assay (CAPA) (Figure 5e) uses a cell line that expresses cytosolic HaloTag protein. HaloTag is a modified bacterial haloalkane dehalogenase that covalently labels itself with a short chloroalkane, and it does so with fast kinetics and high specificity.<sup>222</sup> Cells expressing cytosolic HaloTag protein were incubated with a chloroalkane-conjugated molecule-of-interest. Molecules that reached the cytosol reacted with HaloTag and blocked its active site. Subsequent incubation with chloroalkane-labeled dye resulted in labeling of all unreacted HaloTag, providing a signal that was inversely proportional to the extent of cytosolic localization of the molecule-of-interest.<sup>220,221</sup> CAPA signal was quantified by flow cytometry, allowing quantitation in a 96-well plate format. One downside of this assay is that the signal is inversely proportional to the amount of internalized material, limiting sensitivity for detecting small amounts of material localized to the cytosol.

Cell penetration assays that rely on enzymes are versatile and compartment-specific, and can be very high-throughput. However, assays that do not use native enzymes, such as the biotin ligase assay and CAPA, must use transiently transfected or, preferably, stably transfected cell lines. These assays are also tag-based, and thus careful controls are required to minimize perturbations by the tag and/or limit the extent of signal that might be due to degradation of the cargo.

### **Assays That Measure Cell Death Following Delivery of Toxic Cargo.**

Some reports have used a direct phenotypic readout to measure delivery of a cargo that was toxic to cells, or induced apoptosis.<sup>223,224</sup> Zahaf et al.<sup>225</sup> measured effective translocation of the catalytic part of ADP-ribosyltransferase TccC3 by anthrax toxin by evaluating the

viability of two cancer lines. Quantification has also been accomplished by detecting markers of apoptosis.<sup>226,227</sup> Importantly, analyzing the delivery efficiency with a biological readout must be performed very carefully and with a thorough understanding of the delivery mechanism. Potential effects on cellular integrity by individual components of the delivery system and/or stress induced by the delivery process itself must be excluded to ensure that the biologic effects are solely due to the cytosolic or nuclear presence of the cargo. Finally, there are many factors other than cytosolic localization of the cargo that might alter the health of cultured cells. As a result, assays that measure cell death as a proxy for cytosolic localization can be less specific, and thus less reliable, than other options.

## ARTIFACTS AND MISINTERPRETATIONS IN MEASUREMENTS OF TOTAL CELLULAR UPTAKE AND CYTOSOLIC LOCALIZATION

Maximal cytosolic delivery remains the goal of most delivery systems. Clearly, assays are needed that can unambiguously differentiate between total cellular uptake, which includes material trapped in endosomes, and cytosolic localization. Unfortunately, some assays are employed in a way that leads to artifacts and results in misinterpretation. These are most disruptive to the field when they overestimate the efficiency of a delivery mechanism. In this section, we highlight some common pitfalls and how to avoid them.

### Artifacts and Misinterpretations of Fluorescence Microscopy.

Microscopy is a versatile tool to qualitatively observe the intracellular location of delivered cargo molecules. Many reports have claimed the cytosolic localization of a cargo protein with a preferred delivery method based solely on microscopy using a dye-labeled cargo. In the final analysis, this method requires absolute differentiation of punctate staining from diffuse staining, distinguishing endosomally trapped material from cytosolic material. Both nuclear exclusion and nuclear enrichment can be misinterpreted and actually instead indicate that the cargo has *not* reached the cytosol. Nuclear exclusion can indicate endosomal localization (particularly for cargoes small enough that they should have traversed the nuclear pores if they had accessed the cytosol). Similarly, at low resolution, apparent nuclear enrichment can also instead indicate endosomal localization, since membrane-enclosed vesicles can appear nuclear or perinuclear.

Because the desired outcome is typically to achieve some degree of cytosolic delivery, there is an inherent temptation to interpret diffuse staining as cytosolic localization of a dye-labeled molecule. However, diffuse staining in fluorescence micrographs must be interpreted cautiously, as it can have multiple origins. Something as simple as poor resolution may give the impression of homogeneous staining, but even higher-resolution microscopy can misinterpret out-of-frame fluorescence or very small puncta as diffuse, cytosolic staining. Conversely, only the highest confocal microscopy standards, combined with the most meticulous safeguarding against permeabilization artifacts in preparing and treating microscopy samples, can protect against an erroneous conclusion of delivery to the cytosol.

Prior to the mid-2000s, cells were often fixed prior to observation by fluorescence microscopy, in part to improve resolution. These fixation conditions ranged from mild (4%

paraformaldehyde) to harsh (methanol).<sup>88,230–234</sup> A historically relevant example is the discussion of fixation artifacts in the measurement of the delivery efficacy of VP22, a type I herpes simplex virus tegument protein.<sup>235</sup> Results suggested that a greater degree of internalization was observed with methanol fixation as compared to paraformaldehyde fixation. The authors rationalized this observation by proposing that methanol fixation was able to “concentrate”, “refold”, or “dequench” internalized GFP–VP22 fusion proteins, as compared to the milder fixation by PFA, which was proposed to quench GFP signals.<sup>235</sup> Since that time, it has become clear that the appearance and distribution of intracellular fluorescence is dependent on the fixation method for translocated proteins and oligonucleotides.<sup>236,237</sup> Lundberg et al.<sup>238</sup> further showed the apparent delivery of VP22 before and after fixation with methanol and demonstrated that highly positively charged molecules locate to plasma membranes before fixation and, upon fixation relocate to the nucleus, interact with negatively charged DNA. The same behavior was shown with histone H1, which was found in the nucleus of cells only after fixation.<sup>238</sup>

In 2003 the uptake mechanism of TAT and polyarginine (Arg)<sub>9</sub> was evaluated with respect to fixation conditions. Very different location patterns for translocated CPP were observed in fixed and nonfixed samples.<sup>18</sup> Cells fixed with formaldehyde showed nuclear staining and some cytosolic distribution, but in unfixed cells, the CPPs colocalized mainly with endosomal marker. The authors concluded that the highly charged CPP was bound to the endosomal membrane, and the membrane was disrupted upon fixation. Thus, cytosolic distribution of the peptide observed following fixation was an artifact of the fixation process. This was further underlined by Fischer et al. in 2007,<sup>239</sup> who concluded that membrane integrity is lost upon fixation, and the delivered peptides redistribute and associate with negatively charged DNA.

On the basis of these studies, cells should never be fixed prior to assessing the cytosolic localization of a molecule. While modern assay development avoids fixation altogether, care must be taken in interpreting the literature, particularly older reports that have examined cytosolic or nuclear staining after fixation. Only live cell imaging should be used to examine subcellular localization of a cargo or molecule-of-interest.<sup>240,241</sup> To minimize errors of interpretation, confocal microscopy should be used to eliminate out-of-frame fluorescence, with a resolution high enough to unequivocally differentiate punctate staining from true diffuse, cytosolic staining. Explaining detection after fixation by concentration effects or removal of interfering or quenching components can nowadays not be justified. In general, the authors must rigorously exclude any processes, additives, or conditions that may compromise membrane integrity or demonstrate experimentally that these conditions do not permeabilize membranes.

### **Extremely High Concentrations of Cargo Molecules.**

Another common experimental condition that may contribute to misinterpretation is extremely high concentrations (10  $\mu\text{M}$ , and even 50  $\mu\text{M}$  or above have been reported) of molecules-of-interest.<sup>14,43,242–244</sup> Because of the low efficiency of many delivery methods, it is common for investigators to increase molecule concentrations until an internalized or cytosolic fraction can be detected. Instead of taking low fluorescent signals as a sign of

inefficient delivery to the cytosol, such low signals have also been interpreted as being caused by quenching by other molecules, motivating some investigators to use concentrations as high as 50  $\mu\text{M}$ . For more efficient delivery systems, this should be unnecessary—for instance, Verdurmen et al.<sup>219</sup> showed that cytosolic localization can be detected at low nanomolar concentrations, with outside concentrations in the nanomolar range, when appropriate methods of detection and efficient translocation systems are used.

Delivery mediated by CPPs and supercharged proteins can show evidence of endosomal escape, typically at or above a certain threshold concentration,<sup>36</sup> partly as an effect of endosomal membrane rupture of highly packed endosomes. An attractive model for this effect is that, due to the high net positive charge of CPPs and supercharged proteins, a water influx occurs into the endosomes and leads to the rupture of endosomal membranes, allowing cytosolic diffusion of delivered cargo.<sup>37</sup> The higher the concentration of positively charged molecules, the faster and more efficient is this process.<sup>36,37</sup> To avoid endosomal membrane rupture as a possible mechanism for endosomal leakage, concentrations below 10  $\mu\text{M}$  should ideally be used; if needed, a more sensitive assay should be used to detect the presence of cargo molecules at lower concentrations.

Another problem with increasing concentrations until some degree of penetration is observed is the likelihood of aggregation or insolubility at high concentrations. Even at more reasonable concentrations, molecules of interest can become insoluble, forming aggregates that may or may not be visible as cloudiness or turbidity. Insolubility is often exacerbated by the addition of large, hydrophobic tags. Molecules that are soluble in buffer may also be poorly soluble in culture medium, and this insolubility should not be ignored since cultured cells require growth medium to minimize stress. For all cell penetration experiments, the possibility of molecule insolubility or aggregation should be eliminated by careful preparation of solutions, including filtering and/or centrifugation, and inspection of treated cells using microscopy to check for the presence of aggregates. These practices should be routine to ensure material remains soluble under assay conditions.

If authors intend to show an energy-independent uptake mechanism, delivery experiments are often performed at 4 °C and compared to those at 37 °C. Many authors propose that an alternative, energy-independent uptake mechanism must be occurring if the molecule-of-interest is internalized at 4 °C. Yet, some reports investigated uptake at 4 °C by incubation at concentrations up to 500  $\mu\text{M}$ .<sup>245–247</sup> At such high concentrations, molecules sticking to the cell surface or residual presence of cargo protein after washes can give rise to a signal falsely interpreted as intracellular delivered protein.<sup>75</sup> The interpretation of observed intracellular cargo might thus become questionable and especially so when combined with fixation.<sup>43,246</sup> Careful processing of cells, avoiding sudden temperature changes or similar stress-inducing procedures before or after the incubation with cargo protein, is also crucial to prevent stress-induced rupture and apparent cytosolic delivery.<sup>28,59,63,248</sup> Sudden changes in physiological conditions can potentially alter membrane integrity and/or intracellular organization and should therefore be avoided.

### Medium Components and Cellular Integrity.

Translocation efficiencies have been studied for delivery of specific cargoes in serum-containing medium, serum-free medium and PBS.<sup>28,59,61,242,246,249</sup> Intracellular accumulation of cargo molecules increases upon incubation with serum-free medium or PBS for some CPPs, compared to accumulation in cells treated with serum-containing medium.<sup>242,250</sup> The underlying mechanism is the interaction between the positively charged CPP and negatively charged serum components that inhibit cellular interaction of the CPP.

If cell-based assays are not performed in growth medium, cellular viability and membrane integrity should be evaluated with great care, especially when other additives are used.<sup>248</sup> Dimethyl sulfoxide (DMSO) for instance; has been widely used for cryopreservation of cells and tissue, as a DNA transfection enhancer, as well as an enhancer in topical pharmaceutical formulations.<sup>251–254</sup> Studies suggest that high concentrations of DMSO lead to membrane loosening, pore formation, and even the collapse of the lipid bilayer.<sup>255</sup> While some reports have investigated the effects of high concentrations of DMSO,<sup>246,256,257</sup> if cellular membrane integrity cannot be ensured, it is not possible to evaluate the effectiveness of a delivery mechanism. Even low (1% or less) concentrations of DMSO can lead to phenotypic changes in cultured cells. Because of significant safety considerations DMSO would also not be useable in vivo.

Therefore, ideally a delivery system would be tested in the standard growth medium of the cell line used, with no other additives.

### Clinical Applications of Presented Delivery Mechanisms.

Clinical or in vivo applications of most described delivery mechanisms, notably those relying on positive charges, remain very challenging due to poor pharmacokinetics, nonspecific interactions with serum components and other molecules, lack of cell specificity, endosomal entrapment, and poor efficiency for cytoplasmic delivery of cargo molecules.<sup>27,258–260</sup>

In a recent review, Bernkop-Schnürch<sup>261</sup> pointed out that a common issue for the charge-based delivery system is that during in vivo applications they pass through a gauntlet of polyanions—mucus glycoproteins, proteoglycans, and serum proteins—on the way to their target tissue or organ. He termed this the “polycation dilemma”. If efficient translocation in vitro can only be obtained in the absence of serum, then such a delivery mechanism would be very challenging to develop for any in vivo applications. To solve the “polycation dilemma”, more advanced constructs have been developed, such as cleavable CPPs with an anionic counterpart.<sup>261</sup> However, it is currently unclear whether they can be developed for in vivo use.

As stated above, several delivery systems have been shown to work effectively only above a high threshold concentration. The higher the concentration needed for cytosolic delivery, the lower the efficiency of cellular uptake and endosomal escape. If one only sees cytosolic localization at very high concentrations, one should ask whether the translocation mechanism is truly relevant for potential in vivo applications, or whether it will be limited to in vitro applications.<sup>46</sup> For many biomolecules, it remains questionable whether high

micromolar concentrations can be delivered to tissues of interest, and if a cytosolic localization mechanism that requires such a high concentration could be used in vivo without severe side effects.

Another issue of most delivery systems is the potential for immunogenic effects. VLPs, for instance, have been used as vaccines due to their high immunogenicity. This makes it uncertain whether they would be viable vectors for protein delivery in vivo.<sup>81</sup>

## CONCLUSIONS AND PERSPECTIVES

The toolbox for measuring “cell penetration” is continuously being expanded. We advocate that the consideration of “cell penetration” should be replaced by an emphasis on distinguishing total cellular uptake and cytosolic localization of a cargo. This emphasis acknowledges the reality that a majority of internalized material typically remains trapped in endosomes or in lysosomes and that many desirable biological effects require efficient delivery into the cytosol or nucleus. There is no universal assay for quickly and quantitatively measuring the uptake and cytosolic localization of any cargo, and each method discussed above offers unique advantages and disadvantages. As with all reductionist approaches to biology, making measurements using multiple different assays will be the most reliable way to make firm conclusions about the cytosolic localization of a specific molecule-of-interest or delivery method.

Intracellular biotherapeutics offer great promise, providing distinct solutions to fundamental problems that hamper small molecules. If efficiently delivered to the cytosol, protein drugs would expand the “druggable” proteome far beyond the small subset of intracellular targets that possess a binding pocket for small molecules. RNA or DNA therapeutics, in turn, would be able to modulate many steps that are inaccessible by modulating protein targets. Gene editing complexes would ultimately allow the fundamental reprogramming of a cell’s function. All of these modalities depend on cytosolic or nuclear delivery, but many hurdles remain. Today, the overwhelming majority of delivery strategies have not demonstrated any cell specificity, and some show toxicity. It remains unclear how to widen the therapeutic window if such biomolecules not only interact with most tissues, but also with many serum components. Future research will have to address cell specificity and tissue targeting in a major way.

Upon reading some of the literature of the last 25 years, one might erroneously conclude that delivery to the cytosol is very straightforward. If that were true, there would already be numerous FDA-approved drugs that are based on biomolecules delivered to the cytosol. The degree of difficulty should not dull the excitement surrounding the promise of cytosolically active biotherapeutics, but further progress will rely on quantitative and accurate assessments of biomolecule delivery to the cytosol. The field is not served by misinterpretations, such as inferring cytosolic localization when only total cellular uptake has been measured. Widespread success of therapeutic biomolecules with cytosolic targets will only become a reality if data are generated using robust experiments that unambiguously measure cytosolic localization.



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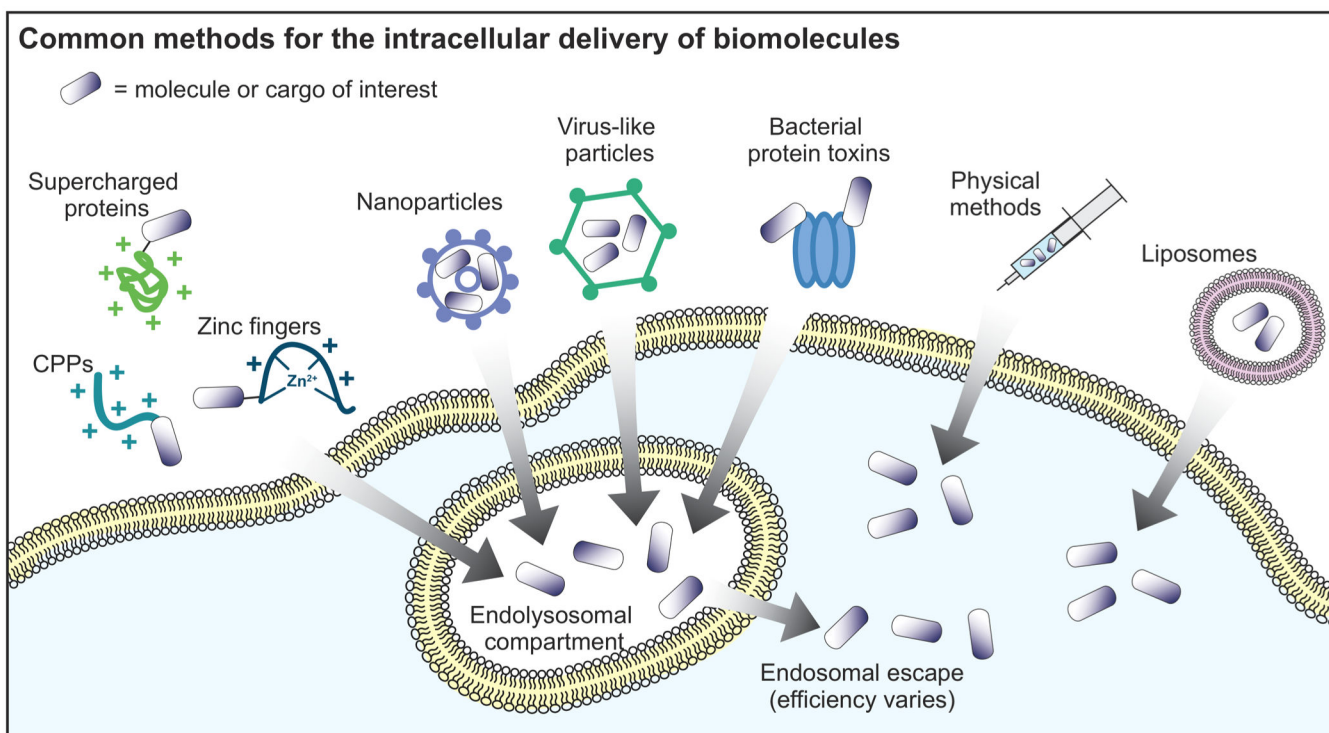
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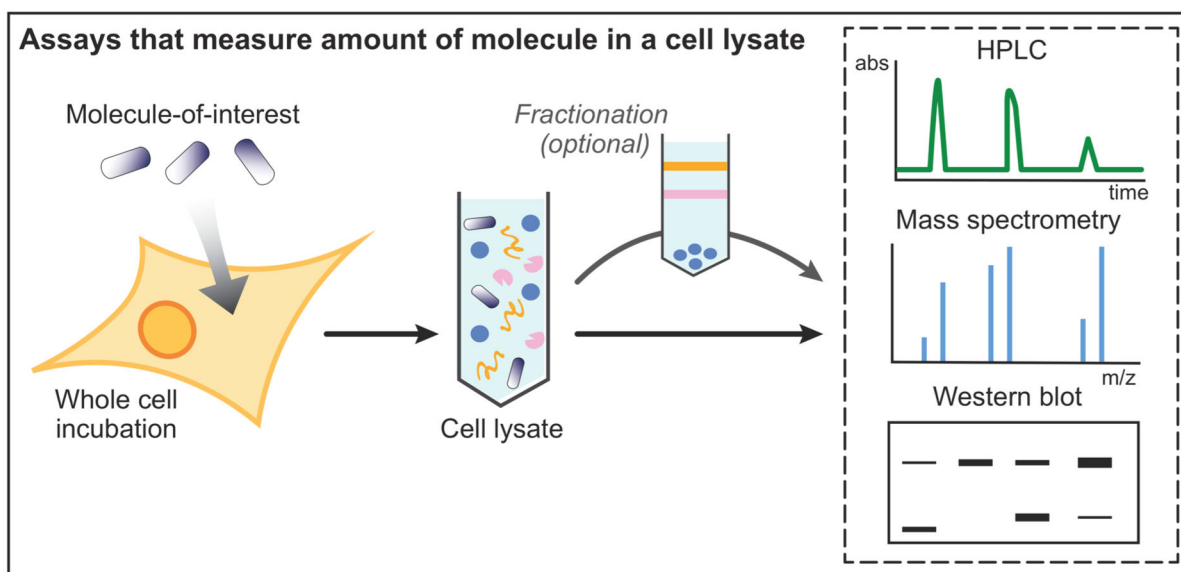
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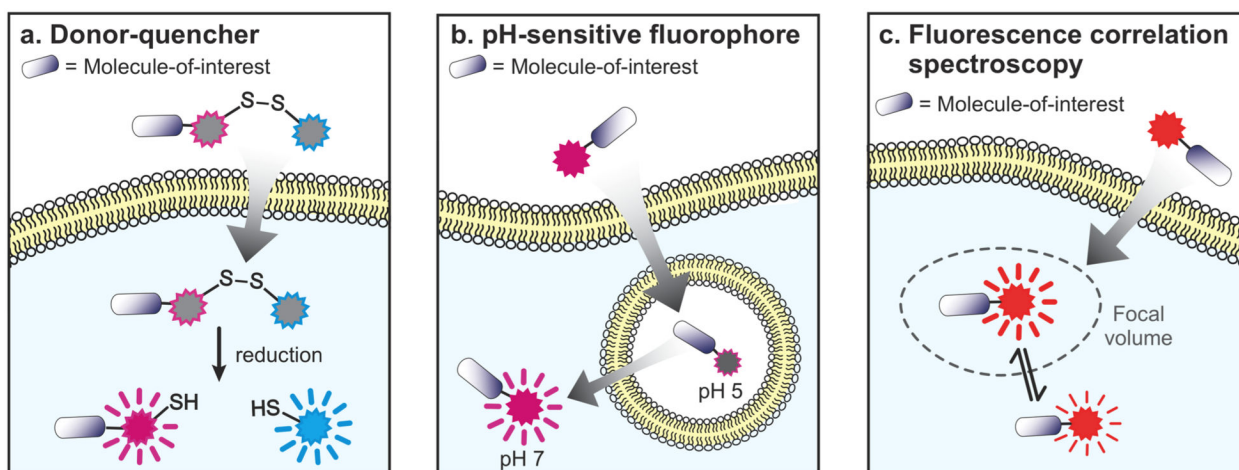
**Figure 1.** Common methods for the intracellular delivery of biomolecules. Cargo molecules can be fused to positively charged molecules, fused to bacterial protein toxins, or packed in nanoparticles or virus-like particles for cellular uptake and eventual endosomal escape. Physical methods and liposomes can directly deliver cargo molecules to the cytosol.



**Figure 2.**

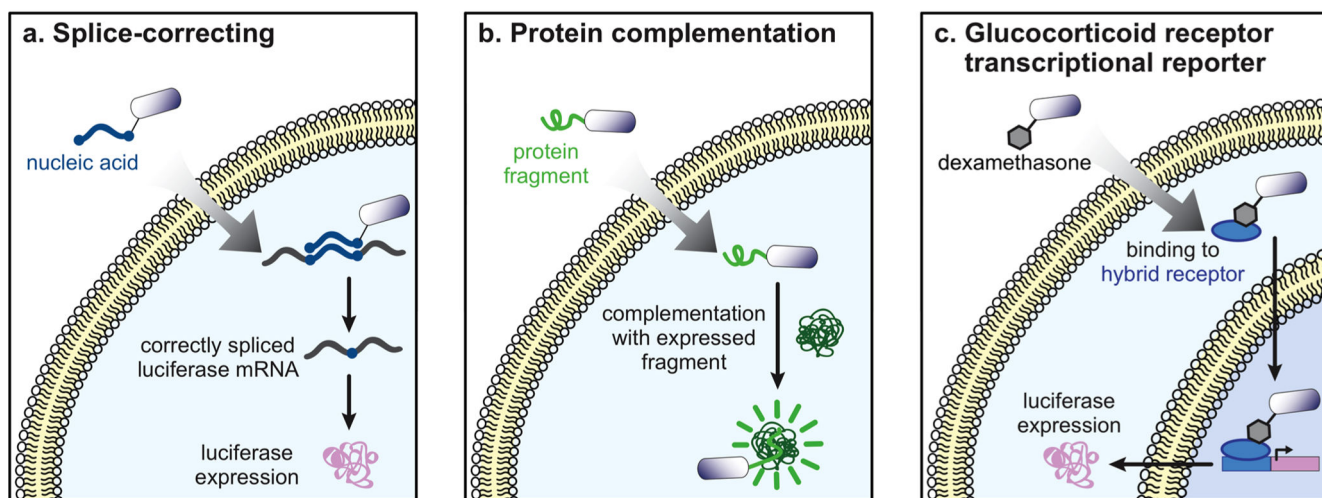
Assays that measure the amount of molecule in a cell lysate. The cells are incubated with the molecule or cargo being investigated, followed by cell lysis. The efficient removal (or their exclusion from the assay) of molecules still bound to the surface is a critical step. Subcellular fractionation is an optional step that can be used to isolate specific subcellular compartments, requiring additional controls for excluding postlysis redistribution. The cell lysate (or fraction of the cell lysate) can then be used in several different assays to quantitate internalized molecule, including HPLC, mass spectrometry, and quantitative Western blotting.





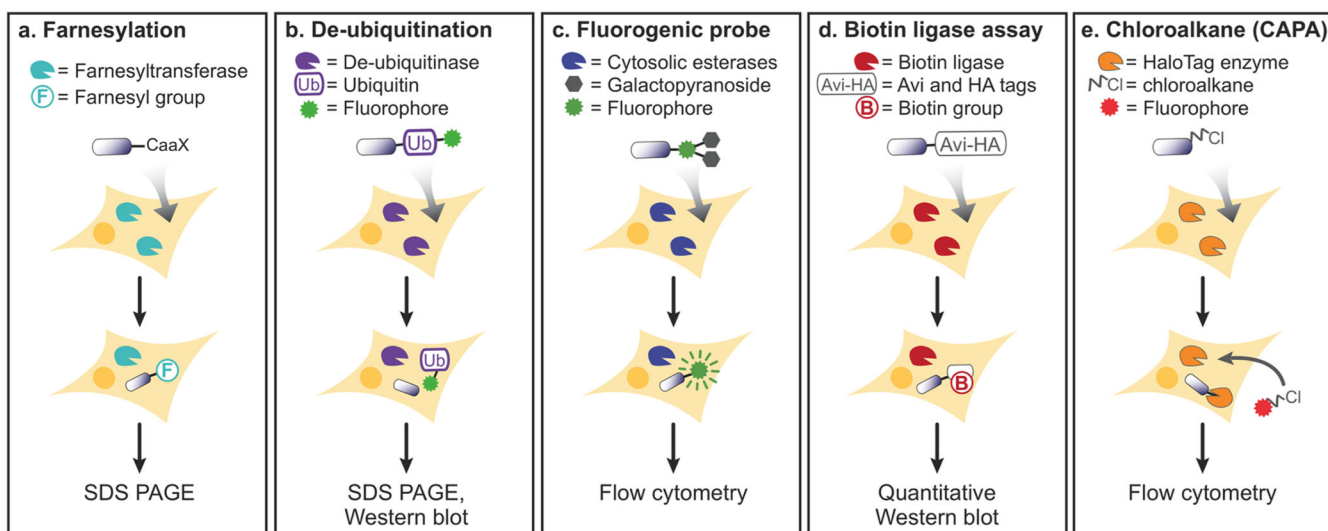
**Figure 3.**

Assays that distinguish cytosolic fluorescence from fluorescence in endosomes or other compartments. (a) If a molecule-of-interest or CPP cargo is labeled with a fluorophore (magenta), and also linked to a fluorescence quencher (blue) via a disulfide bond, then cytosolic localization can be inferred from dequenching of the fluorophore following reduction of the disulfide bond. (b) The pH-sensitive dye naphthofluorescein has low fluorescence in acidic environments such as endosomes and higher fluorescence in the cytosol where pH is close to 7. (c) Fluorescence correlation spectroscopy uses a diffusion model to quantitate absolute concentrations of a fluorescent dye within a focal volume chosen to exclude endosomes and other subcellular compartments. Caveats for each of these methods are summarized in Table 2.



**Figure 4.**

Assays that measure expression of a reporter protein. (a) In the splice-correcting assay, molecules-of-interest are labeled with a nucleic acid sequence that is complementary to an interrupted luciferase mRNA transcript. When the molecule reaches the cytosol, the nucleic acid label corrects the aberrantly spliced transcript, resulting in luciferase expression that can be detected using standard luminescence methods. (b) Molecule-of-interest labeled with dexamethasone can bind to the cytosolic glucocorticoid receptor. This leads to translocation to the nucleus and expression of a reporter gene (luciferase or GFP). (c) A molecule-of-interest labeled with a GFP-derived peptide can reconstitute GFP upon cytosolic localization by complementing a genetically expressed, larger fragment. Caveats for each of these methods are summarized in Table 2.



**Figure 5.**

Assays that measure direct interactions with cytosolic enzymes. (a) The farnesylation assay involves the transfer of a farnesyl group to a CaaX motif appended to the molecule-of-interest. The extent of farnesylation can be monitored by SDS-PAGE or Western blot. (b) In the deubiquitination assay, a dye-labeled ubiquitin is released from the molecule-of-interest by cytosolic deubiquitinases. This change is also measured by SDS-PAGE or Western blot. (c) Using a molecule-of-interest tagged with a diglycosylated fluorescein, cytosolic localization is quantitated based on extent of galactopyranoside cleavage as measured by fluorescent signal. This signal can be detected by flow cytometry since it can only originate from the cytosol. (d) When it enters the cytosol, molecule-of-interest is biotinylated by cytosolic bacterial biotin ligase. The extent of biotinylation can be measured using a quantitative Western blot. (e) Chloroalkane-labeled molecule-of-interest irreversibly labels HaloTag protein expressed in the cytosol. The relative amount of unreacted HaloTag is measured by chasing with a chloroalkane dye and measured by flow cytometry. Caveats for each of these methods are summarized in Table 2.

**Table 1.**

Summary of Methods for Measuring the Total Cellular Uptake of Biomolecules

method	tag	detection method	throughput <sup>d</sup>	caveats <sup>b</sup>	refs
model membranes	none	UV or LC-MS	high	PAS	109
monolayer	none	LC-MS	high	PAS PARA	121, 125
analysis of cell lysate	varies	HPLC	medium	QUANT	129, 134, 138, 139, 141, 155
		MS		LABEL	
		Western blot		DEG	
fluorescence	dye	fluorescence microscopy/flow cytometry	medium/high	DEG	18, 142
SICM/SECM	none	electrochemistry	low	QUANT INST	154

<sup>d</sup>Throughput: Low throughput refers to methods that require multiple readings per cell, processing large bulk quantities of cells, or preparation of cell lysates; medium throughput refers to methods that require one reading per cell, such as simple or automated fluorescence microscopy; high throughput refers to methods that require one reading per cell but can process many cells rapidly, such as flow cytometry.

<sup>b</sup>Caveats: Important caveats and limitations for each assay are noted as follows: measures passive penetration of artificial phospholipid bilayers, not necessarily of cells (PAS); signal can be derived from processes other than cellular uptake, such as paracellular transport or vesicular transcellular transport (PARA); quantitation requires careful calibration (QUANT); requires a tagged or labeled molecule (LABEL); potential artifacts due to degradation (DEG); sophisticated instrumentation, algorithms, and/or data processing required (INST).

**Table 2.**

Summary of Methods for Monitoring the Cytosolic Localization of Biomolecules

method	tag	detection method	throughput <sup>a</sup>	caveats <sup>b</sup>	refs
environment-sensitive fluorescence	dye	fluorescence microscopy/flow cytometry	high	LABEL DEG	160, 163
fluorescence correlation spectroscopy (FCS)	dye	fluorescence microscopy	low	NONCYT LABEL DEG	168, 169
splice-correcting	single-stranded complementary RNA	fluorescence or luminescence spectroscopy	high	INST LABEL DEG CELL	171, 172
dexamethasone reporter genes	dexamethasone	fluorescence or luminescence spectroscopy	high	AMPL LABEL DEG CELL	54, 175
split protein complementation	peptide	fluorescence or luminescence spectroscopy	high	DEG QUANT	183,186-188
farnesylation	CaaX tetrapeptide	SDS-PAGE	medium	CELL LABEL DEG	197
deubiquitination	dye and ubiquitin	SDS-PAGE and fluorimaging	medium	LABEL DEG	198
glucocorticoid-induced eGFP translocation (GIGT)	dexamethasone	fluorescence microscopy	medium	LABEL DEG INST	54
calcein release	none or calcein-AM	fluorescence microscopy/flow cytometry	high	CELL LABEL DEG	209, 210

method	tag	detection method	throughput <sup>a</sup>	caveats <sup>b</sup>	refs
fluorogenic probe and enzyme pair	dye and additional functional group	fluorescence microscopy/flow cytometry	high	LABEL DEG QUANT CELL	200, 228
biotin ligase assay (BtrA)	Avi tag	quantitative Western blot	medium	LABEL DEG CELL LABEL	229
chloroalkane penetration assay (CAPA)	chloroalkane	flow cytometry	high	DEG CELL	220

<sup>a</sup>Throughput: Low throughput refers to methods that require multiple readings per cell, processing large bulk quantities of cells, or preparation of cell lysates; medium throughput refers to methods that require one reading per cell, such as simple or automated fluorescence microscopy; high throughput refers to methods that require one reading per cell but can process many cells rapidly, such as flow cytometry.

<sup>b</sup>Caveats: Important caveats and limitations for each assay are noted as follows: quantitation requires careful calibration (QUANT); requires a tagged or labeled molecule (LABEL); potential artifacts due to degradation (DEG); sophisticated instrumentation, algorithms, and/or data processing required (INST); transfected or stable cell line required (CELL); signal is amplified, and thus is not proportional to amount of molecule translocated (AMPL); noncytosolic material may contribute to assay signal (NONCYT).