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Direct Protein Delivery to Mammalian Cells Using Cell-permeable Cys$_2$-His$_2$ Zinc-finger Domains

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Abstract

Due to their modularity and ability to be reprogrammed to recognize a wide range of DNA sequences, Cys$_2$-His$_2$ zinc-finger DNA-binding domains have emerged as useful tools for targeted genome engineering. Like many other DNA-binding proteins, zinc-fingers also possess the innate ability to cross cell membranes. We recently demonstrated that this intrinsic cell-permeability could be leveraged for intracellular protein delivery. Genetic fusion of zinc-finger motifs leads to efficient transport of protein and enzyme cargo into a broad range of mammalian cell types. Unlike other protein transduction technologies, delivery via zinc-finger domains does not inhibit enzyme activity and leads to high levels of cytosolic delivery. Here a detailed step-by-step protocol is presented for the implementation of zinc-finger technology for protein delivery into mammalian cells. Key steps for achieving high levels of intracellular zinc-finger-mediated delivery are highlighted and strategies for maximizing the performance of this system are discussed.

Video Link

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Introduction

Highly efficient and versatile protein delivery strategies are critical for many basic research and therapeutic applications. The direct delivery of purified proteins into cells represents one of the safest and easiest methods for achieving this.[1,2] Unlike strategies that rely on gene expression from nucleic acids,[3-5] protein delivery poses no risk of insertional mutagenesis, is independent of the cellular transcription/translation machinery and allows for an immediate effect. However, the lack of simple and generalizable methods for endowing cell-penetrating activity onto proteins routinely confounds their direct entry into cells. Current methods for facilitating intracellular protein delivery are based on the use of naturally occurring[6-8] or designed cell-penetrating peptides,[9-12] supercharged transduction domains,[13,14] nanoparticles[15] and liposomes,[16] virus-like particles[17,18] and polymeric microsphere materials.[19] Unfortunately, many of these approaches are hampered by low cellular uptake rates,[20,21] poor stability,[22] inadvertent cell-type specificity,[23] low endosomal escape properties[24] and toxicity.[25] In addition, many protein transduction technologies reduce the bioactivity of the delivered proteins.[14]

Our laboratory previously demonstrated that zinc-finger nuclease (ZFN) proteins — chimeric restriction endonucleases consisting of a programmable Cys$_2$-His$_2$ zinc-finger DNA-binding protein and the cleavage domain of the FokI restriction endonuclease[26-28] — are inherently cell-permeable.[29] This surprising cell-penetrating activity was shown to be an intrinsic property of the custom-designed zinc-finger domain, a DNA-binding platform that has emerged as a powerful tool for targeted genome engineering,[30-32] and considered to be the result of the constellation of six positively charged residues on the protein surface. Indeed, several DNA-binding proteins, including c-Jun and N-DEK have been shown to possess an innate capacity to cross cell membranes.[33] More recently, our laboratory expanded on these results and demonstrated that the cell-penetrating activity of zinc-finger (ZiF) domains could be leveraged for intracellular protein delivery. Genetic fusion of either one- or two-finger ZiF domains to specific protein cargo led to uptake efficiencies that exceeded many conventional cell-penetrating peptide delivery systems.[34] Most notably, ZiF-mediated delivery did not compromise the activity of fused enzymatic cargo and facilitated high levels of cytosolic delivery. Collectively, these findings demonstrate the potential of the ZiF domain for facilitating the efficient and facile delivery of proteins, and potentially more diverse types of macromolecules, into cells.

Here, a detailed step-by-step protocol on how to implement ZiF technology for protein delivery in mammalian cells is presented. We previously constructed a suite of one-, two-, three-, four-, five- and six-finger ZiF domains that lack the ability to bind DNA, due to substitution of each of the α-helical DNA-binding residues, but are capable of delivering proteins into cells[34] (**Figure 1**). The production and transduction of Emerald GFP (EmGFP) into HeLa cells using a two-finger ZiF domain is described. This protocol is extensible to almost any protein capable of soluble

expression in *Escherichia coli* and nearly any mammalian cell type. Expected results are provided and strategies for maximizing the performance of this system are also discussed.

Protocol

1. Cloning

1. Obtain alanine-substituted two-finger ZiF domains that have been sub-cloned into the pET-28 expression vector system and are available upon request (pET-2F-ZiF).[34]
2. PCR amplify EmGFP from the plasmid Emerald-pBAD with the primers 5' XmaI-EmGFP (5'-GGAAATTG**CCCGGG**ATGGTGAGCAAGGGCGAGGAGCTGTTCAC-3'; *Xma*I site in bold) and 3' SacI-EmGFP (5'-CGGATCT**GAGCTC**TTACTTGTACAGCTCGTCCATGCCGAG-3'; *Sac*I site in bold).
    1. Use 5 ng of template DNA, 10 µl of 10x polymerase buffer, 1 Units (U) of Taq DNA polymerase, 0.2 mM each dNTP and 0.2 µM of each primer in a 100 µl solution with the remaining volume made up of distilled/deionized water. Use the cycling conditions: 95 °C for 5 min; 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min; 72 °C for 10 min. Purify the PCR product by gel extraction and determine DNA concentration using a spectrophotometer measuring Abs_{260} x 50 µg/ml.
3. Digest pET-2F-ZiF and the insert encoding EmGFP with the restriction enzymes *Xma*I and *Sac*I in recommended buffer for 3 hr at 37 °C using 10 U of enzyme per 1 µg of DNA. Visualize DNA by agarose gel electrophoresis using a fluorescent intercalating dye, such as ethidium bromide.
4. Purify the digested DNA by gel extraction kit and determine DNA concentration by a spectrophotometer measuring Abs_{260} x 50 µg/ml.
5. Ligate the purified EmGFP-encoding DNA into 50-100 ng of pET-2F-ZiF using 1 U of T4 DNA Ligase for at least 1 h at RT. For best results, perform the ligation reaction using a 6:1 insert-to-vector molar ratio.
6. Thaw 50 µl of any chemically competent XL-1 Blue *Escherichia coli* cells on ice and mix gently with 10-20 ng of ligated pET-2F-ZiF-EmGFP.
7. Keep on ice for 30 min. Heat shock the mixture at 42 °C for 90 sec and recover the cells in 2 ml of Super Optimal broth with Catabolite repression (SOC) for 1 hr at 37 °C with shaking.
8. Spread 100 µl of recovery culture on a lysogeny broth (LB) agar plate with 50 µg/ml kanamycin and incubate O/N at 37 °C.
9. The following day, inoculate 6 ml of Super Broth (SB) or LB culture containing 50 µg/ml kanamycin with one colony from the LB agar plate and culture O/N at 37 °C.
   Note: Colony PCR using the primers 5' XmaI-EmGFP and 3' SacI-EmGFP could be used to identify positive clones prior to miniprep.
10. Purify pET-2F-ZiF-EmGFP by miniprep and confirm plasmid by DNA sequencing using T7 promoter (5'-TAATACGACTCACTATAGGG-3').

2. Expression and Purification

1. Thaw 50 µl of chemically competent BL21 *E. coli* cells on ice and mix gently with 100 ng of pET-2F-ZiF-EmGFP plasmid. Transform as described in steps 1.7-1.8.
2. The following day, inoculate 10 ml of LB medium containing 50 µg/ml kanamycin with a single colony and grow O/N at 37 °C with shaking.
3. The following day, dilute the 10 ml of O/N culture into 1 L of LB medium supplemented with 50 µg/ml kanamycin, 0.2% glucose and 100 µM $ZnCl_2$. Grow the culture at 37 °C with shaking to an optical density at 600 nm (OD_{600}) of 0.8 and induce protein expression with 2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 6 hr of growth at 37 °C, harvest cells by centrifugation at 5,000 x g for 10 min at 4 °C.
   Note: Induction conditions are highly variable and depend on the stability of the proteins being expressed. Monitor the OD_{600} every 30 min until an OD_{600} of 0.8 is reached.
4. Resuspend the cell pellet in 5 ml of lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 100 µM $ZnCl_2$, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM imidazole, pH 8.0). Lyse the cells on ice by sonication with the following setting: 50% power output, 4 min process time with 5 sec on/10 sec off intervals. Avoid overheating the solution.
5. Centrifuge the cell lysate at 25,000 x g for 30 min at 4 °C and transfer the supernatant into a fresh collection tube. For best results, perform all the following steps at 4 °C.
6. Run the supernatant through a column pre-packed with 1 ml of equilibrated Ni-NTA slurry. Wash the resin with 20 ml of wash buffer (50 mM Tris-HCl, 500 mM NaCl, 100 µM $ZnCl_2$, 1 mM DTT, 1 mM $MgCl_2$ and 30 mM imidazole, pH 8.0).
7. Elute the protein with 5 ml of elution buffer (50 mM Tris-HCl, 500 mM NaCl, 100 µM $ZnCl_2$, 1 mM DTT, 1 mM $MgCl_2$, and 300 mM imidazole, pH 8.0).
8. Buffer exchange the eluted protein with storage buffer (50 mM Tris-HCl, 500 mM NaCl, 100 µM $ZnCl_2$, 1 mM DTT, 1 mM $MgCl_2$ and 10% glycerol, pH 8.0) and concentrate the protein to at least 40 µM using a spin concentrator by centrifugation according to the manufacturer's instructions.
9. Determine protein concentration by BCA or Bradford assay. Mix 2 µl of purified proteins with 2 µl 2× SDS-PAGE loading dye, boil at 95 °C for 10 min and then resolve on 4%-20% Tris-Glycine SDS-PAGE to assess protein purity (**Figure 2**).

3. Protein Storage

1. Aliquot the concentrated protein, flash freeze in liquid nitrogen and store at -80 °C. For EmGFP fusion proteins, cover the tube with aluminum foil in order to protect the protein from light.
2. Avoid repeated freezing and thawing of the protein solution. Note: ZiF-fused proteins are stable under these conditions for at least 1 month. Inappropriate or >4 month storage may lead to protein precipitation or photobleaching of EmGFP.

4. Protein Transduction

1. Maintain HeLa cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic-antimycotic at 37 °C in a fully humidified atmosphere with 5% CO_2.
   Note: Cells passaged more than 30 times are not recommended for protein transduction.
2. Pre-coat a 24-well plate with 500 µl of 50 µg/ml of poly-lysine for 30 to 60 min at 25 °C. Seed cells onto a 24-well plate at a density of 2×10^5 cells per well. At 24 hr after seeding, or once cells are between 80%-90% confluent, remove media from each well and wash with 500 µl of pre-warmed serum-free DMEM (SFM).
3. To each well, add 250 µl of SFM containing 2 µM of ZiF-EmGFP protein and 100 µM $ZnCl_2$. Incubate cells at 37 °C for 1 hr. Note: ZiF domains enter cells primarily through macropinocytosis,[34] which is an energy-dependent mechanism. Therefore, cells must be incubated at 37 °C for efficient protein internalization.
4. Remove media from cells and wash three times with 500 µl of calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.5 mg/ml of heparin. Note: Heparin is necessary to remove surface-bound protein that might complicate downstream analyses.
5. (Optional) Repeat treatments up to three times for enhanced delivery.
6. Isolate treated cells immediately after heparin wash by digestion with 0.05% trypsin-EDTA at 37 °C for 2 min. Re-suspend detached cells in 0.5 ml of DPBS supplemented with 1% FBS.
7. Measure the fluorescence intensity of each sample by flow cytometry[35] using the fluorescein isothiocyanate (FITC) channel (**Figure 3**). Adjust the forward scatter (FSC) and side scatter (SSC) to place the population of interest on scale, ensuring that populations with different properties are resolved from each other.
8. Collect 10,000 live events for each sample and analyze the data using flow cytometry data analysis software.[36] Normalize fluorescence from HeLa cells treated with ZiF-EmGFP to those cells treated only with SFM.

Representative Results

Two-finger ZiF-EmGFP fusion proteins can be expressed in *E. coli* with >95% homogeneity and high yields (>25 mg/ml) (**Figure 2**). In general, one- and two-finger ZiF fusion proteins can be produced in quantities nearly identical to those of wild-type unmodified protein. However, in some contexts, five- and six-finger ZiF fusion proteins are unable to be produced in yields high enough for downstream applications.

Direct application of two-finger ZiF-EmGFP protein onto HeLa cells for 90 min at 37 °C leads to a dose-dependent increase in EmGFP fluorescence (**Figure 3A**). Critically, no fluorescence is observed in the absence of the ZiF domain. We previously observed that nearly 100% of cells are fluorescent after treatment with only 2 µM of two-finger ZiF-EmGFP protein, and that HeLa cells treated with ZiF fusion protein are positive for EmGFP fluorescence at protein concentrations as low as 0.25 µM (**Figure 3B**).
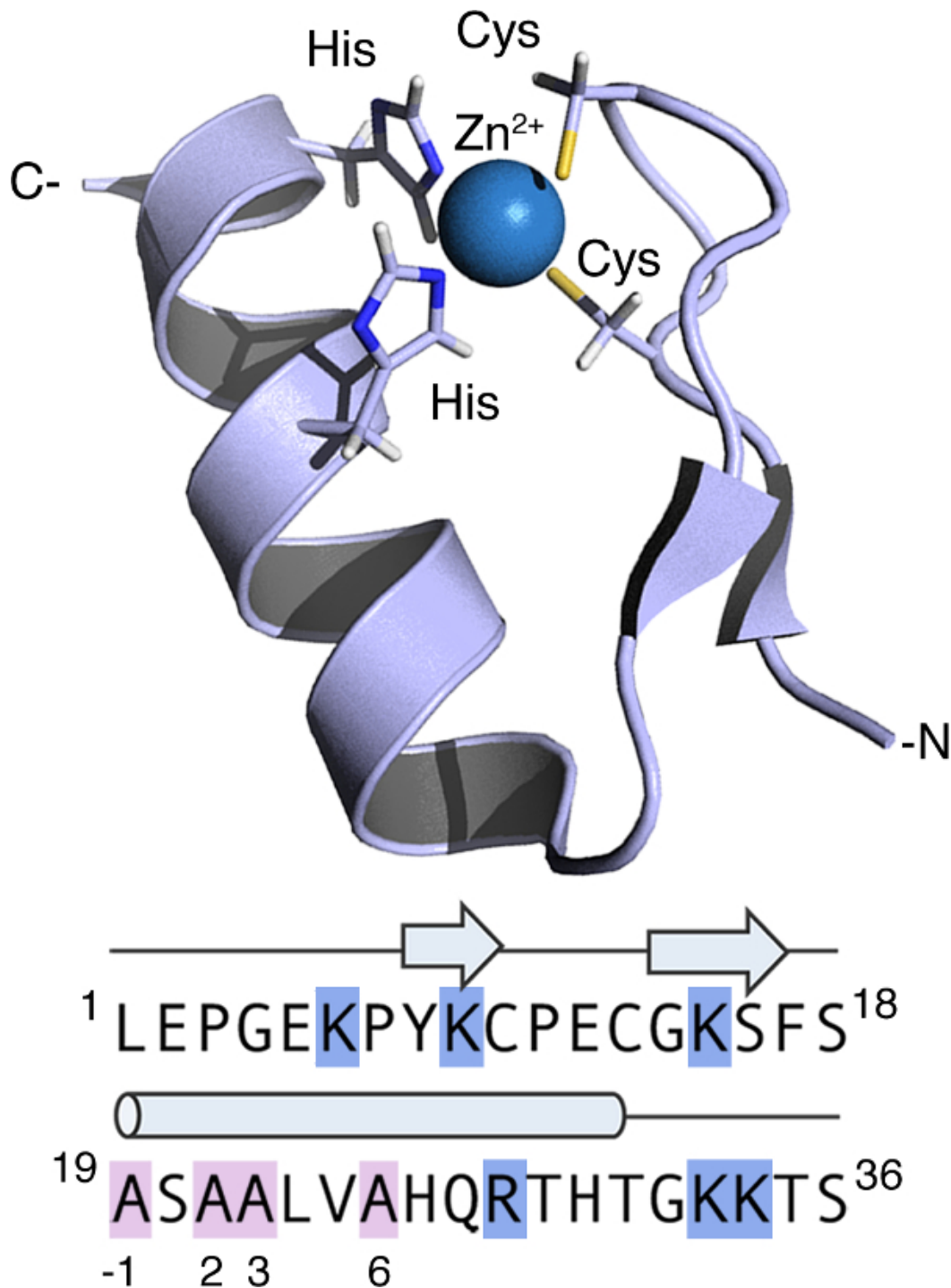
Figure 1. Structure and sequence of zinc-finger protein. (Top) Crystal structure of a single zinc-finger (ZiF) domain. The side chains of the conserved Cys and His residues coordinated with the Zn^{2+} ion are shown as sticks (PDB ID: 2I13).[37] **(Bottom)** Sequence of the ZiF domain. Arrows and cylinders indicate ß-sheet and α-helix secondary structures, respectively. The α-helical DNA-binding residues that have been substituted with alanine are highlighted pink. Positively charged residues predicted to mediate cell internalization are highlighted light blue. Please click here to view a larger version of this figure.
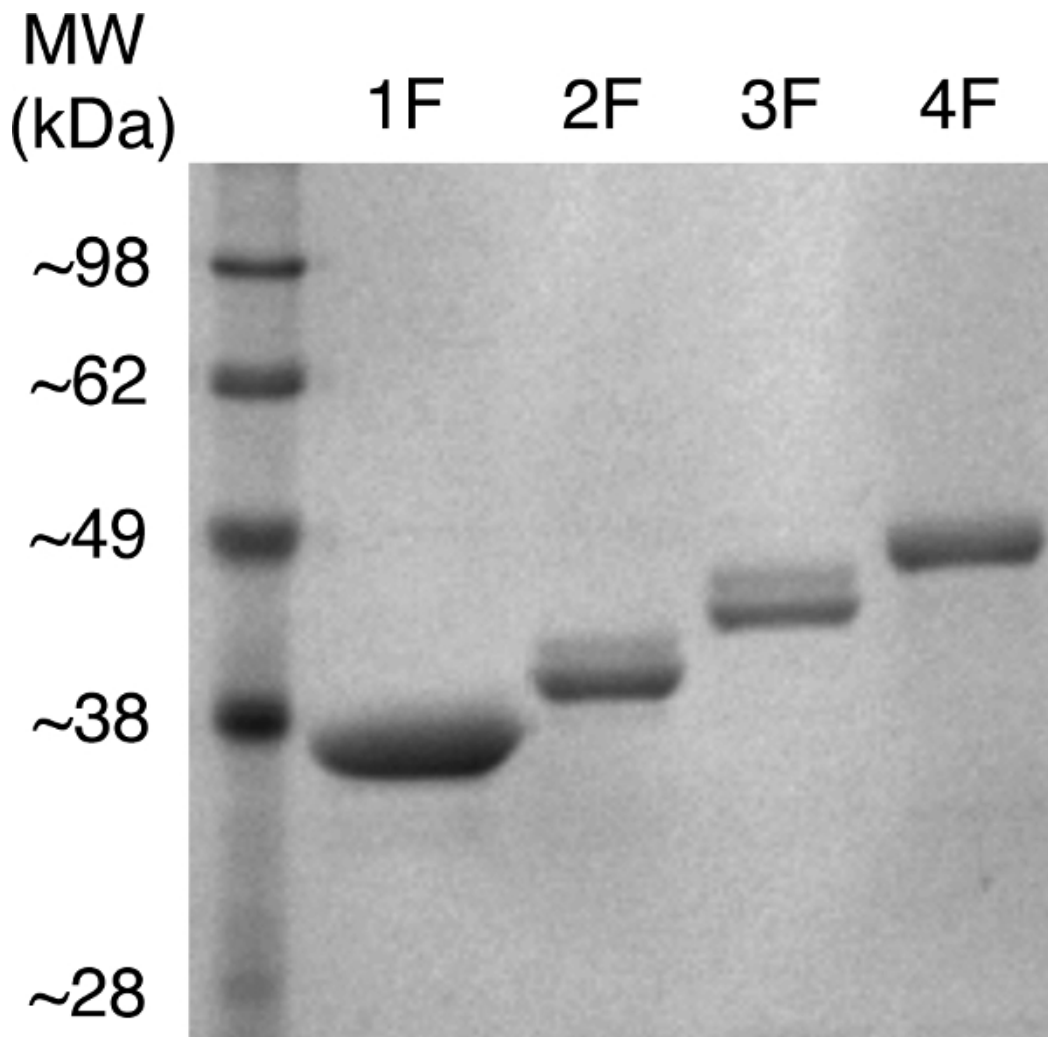
Figure 2. SDS-PAGE of purified one-, two-, three- and four-finger ZiF-EmGFP fusion proteins. ZiF-EmGFP fusion proteins were expressed in *E. coli* and purified by Ni-NTA agarose resin. Eluted protein was analyzed for purity by SDS-PAGE using a 4%-20% Tris-Glycine gel. No significant degradation or truncations of ZiF-EmGFP fusion proteins was observed.
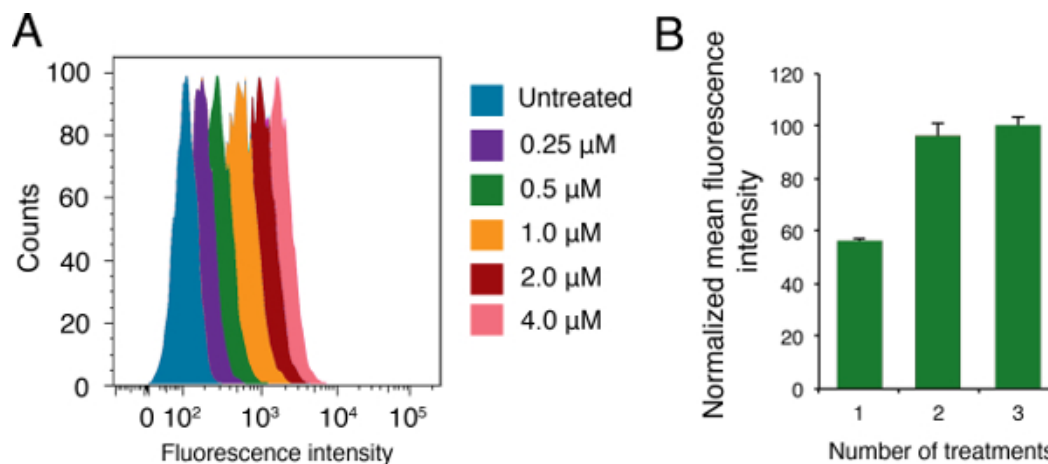

Figure 3. ZiF-mediated protein delivery into HeLa cells. (A) Fluorescence intensity of HeLa cells treated with increasing amounts of two-finger ZiF-EmGFP protein. HeLa cells treated with EmGFP protein alone overlap entirely with untreated cells. **(B)** Normalized fluorescence intensity of HeLa cells treated consecutively with 2 µM of two-finger ZiF-EmGFP protein. Fluorescence intensity was determined by flow cytometry.

Discussion

Here, a step-by-step protocol for protein delivery using cell-permeable zinc-finger (ZiF) domains is presented. The ZiF domain does not reduce the activity of fused enzymatic cargo[34]; allows for the production and purification of proteins in yields nearly identical to those observed with unmodified protein; and can transport proteins and enzymes into a wide range of cell types with efficiencies that exceed traditional cell-penetrating peptide or protein transduction domain systems. Together, these findings indicate the broad potential of ZiF domains for mediating direct protein delivery into cells for a wide range of applications.

Maximum protein delivery was previously achieved using only a two-finger ZiF domain, despite the fact that extended arrays of three- and four-finger ZiF domains carry greater positive charge. These findings indicate that ZiF-mediated cell entry might be influenced by factors other than charge, including protein stability or conformational rigidity. ZiF domain-mediated protein delivery was also found to be energy-dependent and thus requires that all cells treated with protein be incubated at 37 °C. Through the usage of small molecule inhibitors of various endocytic pathways, macropinocytosis, and to a lesser extent caveolin-dependent endocytosis, were determined to be the major pathways for ZiF-mediated cell entry.[34] Notably, unlike other protein transduction systems, ZiF domains are capable of efficiently escaping endosomes to mediate high levels of cytosolic delivery of the fused macromolecular cargo, underscoring the potential of these domains for achieving robust protein delivery.

In our experience, the seeding density of cells is a critical step for achieving high levels of protein transduction. We recommend treating cells once they reach 80%-90% confluency and previously observed that cells seeded at >95% confluency show sub-optimal transduction capacity, while cells seeded at low densities (<50%) are susceptible to protein-induced toxicity. Importantly, for cell types with high detachment tendencies, cell culture plates pre-coated with poly-lysine are recommended. Poly-lysine facilitates cell attachment through electrostatic interactions with negatively charged cell-surface components. Although the α-helical DNA-binding residues of each ZiF domain have been removed to eliminate any potential for DNA recognition, the cysteine and histidine residues that coordinate with the Zn^{2+} ion to stabilize the BBα ZiF domain fold remain intact. Thus, we recommend that any storage buffer be supplemented with at least 100 μM of $ZnCl_2$ to maintain protein integrity.

Although the ZiF domain was previously shown to deliver proteins and enzymes into a variety of cell types, the efficiency of ZiF delivery might also be dependent on the both macromolecular cargo and protein concentration. For instance, cells treated with two-finger ZiF-luciferase fusion protein were observed to display maximum luminescence when treated with 0.5 μM protein, with decreased activity at higher concentrations, while cells treated with two-finger EmGFP exhibited a dose-dependent increase in fluorescence intensity up to 8.0 μM protein, and upon consecutive protein treatments. We therefore recommend evaluating the cell penetrating ability of each unique ZiF domain fusion across a range of concentrations.

Finally, although not yet demonstrated, we anticipate that ZiF domain delivery is a highly flexible platform, capable of delivering a diverse array of macromolecules into cells. For example, it may be possible for both DNA and RNA to be chemically functionalized onto the surface of the ZiF domain via a hydrolyzable linker, or transiently transfected into cells by encapsulation of ZiF domains. Additionally, the efficiency of ZiF protein delivery could be further enhanced by rational design efforts focused on surface charge optimization.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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