

Cell-Type-Specific Intracellular Protein Delivery with Inactivated Botulinum Neurotoxin

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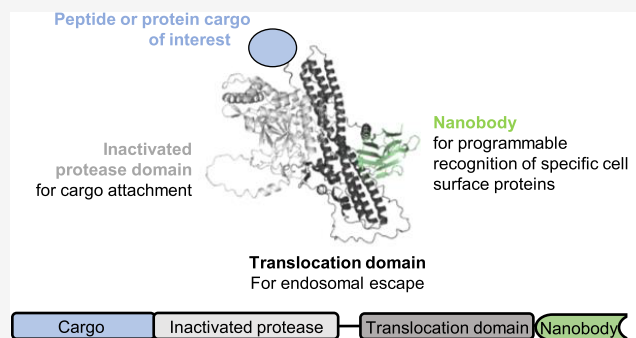


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ABSTRACT: The ability to deliver proteins and peptides across the plasma membrane into the cytosol of living mammalian cells would be highly impactful for both basic science and medicine. Natural cell-penetrating protein toxins have shown promise as protein delivery platforms, but existing approaches are limited by immunogenicity, lack of cell-type-specificity, or their multi-component nature. Here we explore inactivated botulinum neurotoxin (BoNT) as a protein delivery platform. Using split luciferase reconstitution in the cytosol as a readout for endosomal escape and cytosolic delivery, we showed that BoNT chimeras with nanobodies replacing their natural receptor binding domain can be selectively targeted to cells expressing nanobody-matched surface markers. We used chimeric BoNTs to deliver a range of cargo from 1.3 to 55 kDa in size, and demonstrated selective delivery of orthogonal cargoes to distinct cell populations within a mixed culture. These explorations suggest that BoNT may be a versatile platform for targeted protein and peptide delivery into mammalian cells.



INTRODUCTION

Delivery of large macromolecules across the plasma membrane into the cytosol of mammalian cells remains a formidable challenge for both medicine and basic science. Antibodies and other biologics have well-established clinical utility against cell surface and extracellular targets, but they have generally not been extended to intracellular targets due to their inability to cross the plasma membrane. In basic science, the use of large non-genetically encoded biophysical probes such as fluorophore-conjugated proteins or nanoparticles for the study of intracellular signaling has also been stymied by the lack of efficient methods for delivering such probes into the cytosol of living cells.

Because of the central importance of this problem, many different strategies for cytosolic delivery have been explored, and recent years have seen an explosion in lipid nanoparticle^{1,2} and engineered virus-like particles^{3,4} delivery platforms in particular. These technologies appear promising especially for the delivery of oligonucleotide-type cargo, although they largely lack cell-type-specificity. For delivery of peptides and small proteins, cell-penetrating peptides^{5,6}, folded miniproteins,^{7,8} and supercharged proteins^{9,10} have gained traction, although much of the cargo remains stuck in endosomes, causing toxicity and contributing background in some cases.^{11,12}

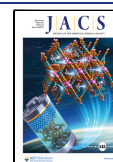
Apart from these strategies, engineered toxins have emerged as a promising platform for the delivery of protein and peptide cargo into cells. A diverse array of toxins has evolved efficient strategies for receptor-mediated endocytosis followed by

escape/translocation through endosomal membranes into the cytosol. Three major toxin classes have been explored as protein delivery platforms—anthrax toxin, diphtheria toxin, and botulinum neurotoxin—each with respective benefits and disadvantages. For example, the anthrax platform is modular and has been used to deliver Ras/Rap1-specific endopeptidase (RRSP) and the A-chain of diphtheria toxin using scFv- or IgG-mediated cell surface binding.^{13,14} However, the multi-component nature of the anthrax platform limits its utility and robustness. Engineered diphtheria toxin has been used to deliver a range of cargo (alpha-amylase,¹⁵ purine nucleoside phosphorylase,¹⁶ and RRSP¹⁷), but immunogenicity is a concern, as many individuals are vaccinated against diphtheria, and general reprogramming of cell-type-specificity with artificial receptor binding domains has not yet been demonstrated.

We were intrigued by the botulinum neurotoxin (BoNT) platform in particular,^{18–21} because humans are not vaccinated against this toxin, reducing its immunogenicity; it is a single-component system; and the toxin's natural cargo—a protease that cleaves the synaptic vesicle fusion proteins SNAP25 and

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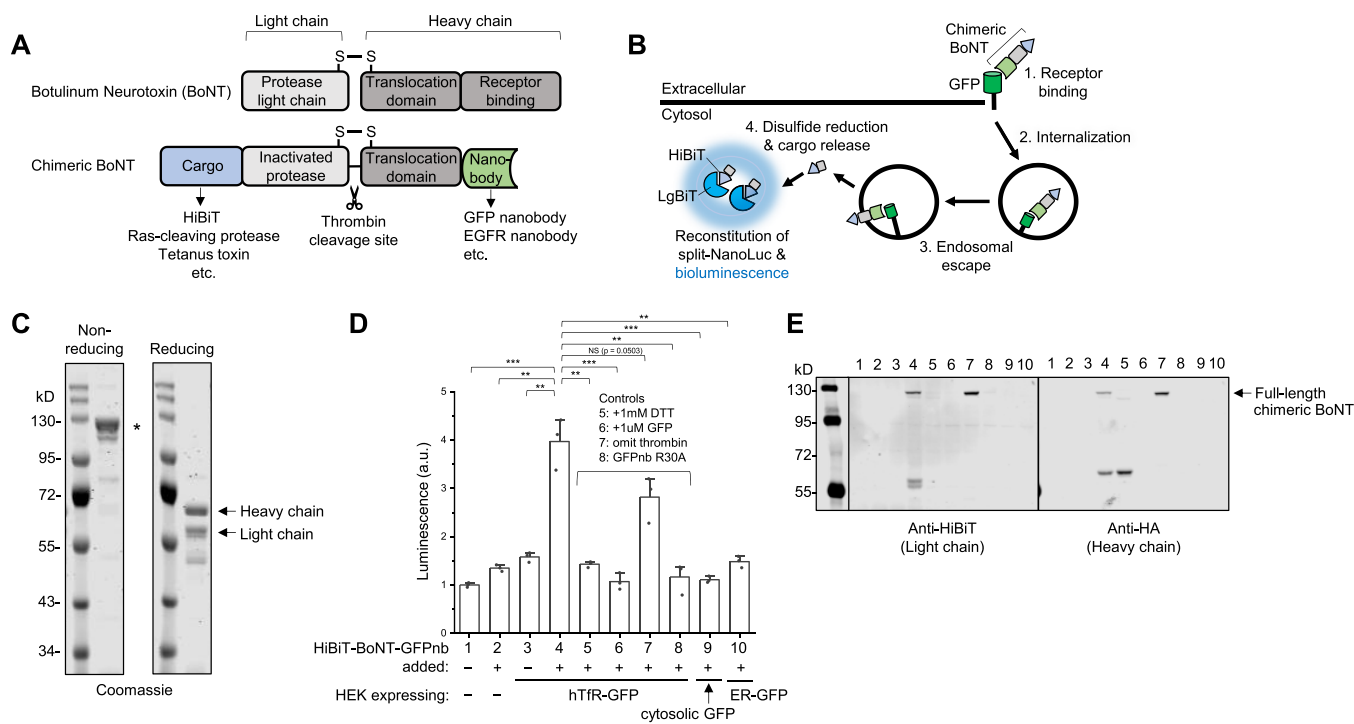


Figure 1. Design of chimeric botulinum neurotoxins (BoNTs) and split-luciferase assay for detecting cytosolic cargo delivery. (A) Structure of wild-type BoNT and chimeric BoNTs. The light-chain protease is inactivated by three mutations (E228Q/R360A/Y363F). The receptor binding domain is replaced by a nanobody, which bears a C-terminal HA epitope tag. Protein cargo is fused at the N-terminus. (B) Schematic of chimeric BoNT binding to surface GFP-expressing mammalian cells and delivering HiBiT cargo to the cytosol. HiBiT reconstitutes with cytosolically expressed LgBiT to generate luciferase activity and bioluminescence in the presence of its substrate, furimazine. (C) Coomassie-stained SDS-PAGE gel of purified recombinant HiBiT-BoNT-GFPnb (GFP nanobody) with and without reduction by DTT. * denotes full-length HiBiT-BoNT-GFPnb. (D) Bioluminescence readout from living cells expressing the indicated constructs and treated with 10 nM of HiBiT-BoNT-GFPnb for 24 h. All HEK293T cells expressed mCherry-LgBiT in the cytosol. *P*-values were calculated using unpaired two-tailed *t* test. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). (E) Western blot analysis of cell lysates from (D) under non-reducing conditions. Released HiBiT cargo (55 kDa) is detected only in sample 4.

VAMP2—has been re-engineered to cut alternative substrates.²² Furthermore, a recent study utilizing BoNT for trans-synaptic tracing in flies²³ suggests that the receptor binding domain of BoNT may be replaceable, opening the door to cell-type-specific delivery. In this work, we explore BoNT as a protein delivery platform, developing a sensitive bioluminescence assay to detect cargo delivery into the cytosol. We demonstrate delivery of several distinct protein cargoes and replace the receptor binding domain with nanobodies to achieve cell-type-specificity.

RESULTS

BoNTs have three domains (Figure 1A, top): a receptor binding domain that enables receptor-mediated endocytosis, a translocation domain for escape from endosomes, and a zinc metalloprotease which is BoNT's natural cargo. BoNT's protease cuts either SNAP25 or VAMP2, which are both essential for synaptic vesicle fusion, thereby inhibiting synaptic transmission in the brain. Four different serotypes of BoNT (A, C, D, and X) have been used in protein delivery research.^{18–21} For our study, we selected BoNT/X,²⁴ because its protease-dead triple mutant (E228Q/R360A/Y363F) has the least *in vivo* toxicity compared to other BoNT serotypes.^{19,25}

To begin, we sought to develop a highly specific and sensitive assay for BoNT-mediated delivery of cargo into the cytosol of living mammalian cells. Previous assays either require cell lysis,^{13,14,16–20} which can produce artifactual

results^{26,27}, or require a specialized instrumentation to analyze cargo diffusion properties in the cell.^{28,29} We wished to develop a live-cell assay that could unambiguously distinguish between successful cytosolic delivery and trapping within endosomes, using simple and widely accessible plate reader-based readout. We also required high sensitivity to detect small quantities of delivered cargo. We selected the split-luciferase system NanoBiT for our assay³⁰ because the fragments can be genetically targeted to specific cellular subcompartments, reconstitution occurs rapidly and with high affinity ($K_d = 700$ pM), and the bioluminescence readout is amplified and therefore highly sensitive.

To test the specificity of the NanoBiT reporter, we prepared HEK293T cells expressing the large fragment of NanoBiT, called LgBiT, in the cytosol. We also transduced the cells with the small fragment (HiBiT) targeted to the cytosol, endoplasmic reticulum (ER) lumen, or cell surface. As expected, bioluminescence was only detected for the cytosol co-localized combination, and not for cell surface-HiBiT or ER-HiBiT samples (Supplementary Figure 1). This suggests that our assay will be able to faithfully detect cytosolic delivery of HiBiT fused to our cargo of interest (Figure 1B).

We then proceeded to design a BoNT variant in which the protease is inactivated and 1.3 kDa HiBiT is fused to its N-terminus (Figure 1A, bottom). We also replaced the receptor binding domain of BoNT with a high-affinity ($K_d = 1$ nM) nanobody against green fluorescent protein (GFP; GFPnb), which bears a C-terminal HA epitope tag.³¹ This engineered

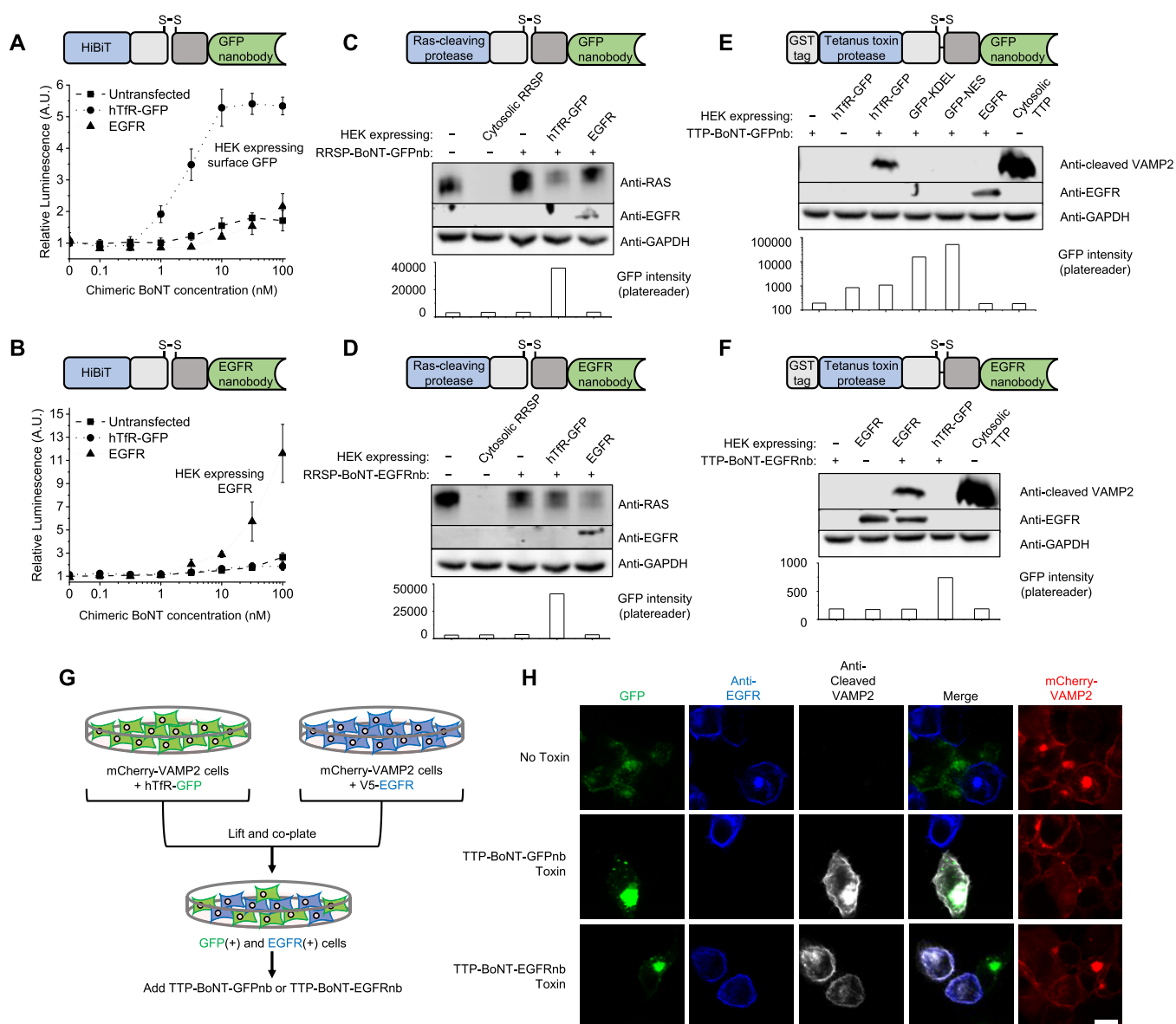


Figure 2. Chimeric BoNT delivers various cargo to mammalian cells in a receptor-dependent manner. (A,B) Delivery of HiBiT-BoNT-GFPnab (A) or HiBiT-BoNT-EGFRnab (B) chimeric toxins to HEK293T cells expressing surface GFP (hTfR-GFP) or EGFR. Bioluminescence reflects HiBiT (cargo) reconstitution with mCherry-LgBiT in the cytosol. Each condition is plotted using the average value of technical replicates ($n = 3$). (C,D) Delivery of Ras/Rap1-specific endopeptidase (RRSP) to the cytosol of GFP-expressing or EGFR-expressing HEK293T cells, using chimeric toxins with GFPnab (C) or EGFRnab (D). HEK293T cells were incubated for 24 h with 25 nM RRSP-BoNT-GFPnab (C) or 100 nM RRSP-BoNT-EGFRnab (D). Lysates were blotted with antibodies against pan-Ras, EGFR, and GAPDH (a cytosolic protein control). An expression plasmid encoding cytosolic RRSP was used as a positive control. GFP fluorescence was measured in live cells before cell lysis. (E,F) Delivery of tetanus toxin protease (TTP) to the cytosol of GFP- or EGFR-expressing cells, using chimeric toxins with GFPnab (E) or EGFRnab (F). All HEK293T cells expressed mCherry-VAMP2, whose cleavage was detected with anti-cleaved VAMP2 antibody. Cells were incubated for 24 h with 100 nM of TTP-BoNT-GFPnab (E) or 100 nM of TTP-BoNT-EGFRnab (F). An expression plasmid encoding cytosolic TTP was used as a positive control. (G) Experimental design for chimeric BoNT delivery to a mixed culture of surface GFP- and EGFR-expressing VAMP2 reporter HEK293T cells. (H) Confocal fluorescence imaging of (G). Cells were treated with 100 nM of TTP-BoNT-GFPnab or TTP-BoNT-EGFRnab for 24 h, and subsequently fixed and stained with anti-cleaved VAMP2 and anti-EGFR antibodies. In the second row, cleaved VAMP2 is only detected in GFP+ green cells, while in the third row, cleaved VAMP2 is only detected in EGFR+ blue cells. Scale bar, 10 μm .

BoNT variant was expressed in *E. coli* and purified using a glutathione S-transferase (GST) tag. The recombinant protein was treated with thrombin protease both to remove the GST tag and to cleave the toxin into “heavy chain” and “light chain” components connected via a disulfide bridge (Figure 1A, bottom). The SDS-PAGE gel in Figure 1C shows the full-length recombinant toxin (119 kDa) and its reduced fragments (55 and 64 kDa) after dithiothreitol (DTT) treatment.

We prepared HEK293T cells stably expressing mCherry-LgBiT in the cytosol and introduced by transient transfection surface GFP fused to the transferrin receptor (hTfR-GFP), which is known to cycle constitutively through endosomes.^{23,32} Incubation with 10 nM chimeric HiBiT-BoNT-GFPnab for 24 h resulted in bioluminescence when furmazine, NanoBiT’s small-molecule substrate, was added to the live cells (Figure 1D). By contrast, bioluminescence was not detected when chimeric

BoNT was not supplied or cells did not express surface GFP. If the HEK293T cells expressed GFP in the cytosol or ER lumen instead of the cell surface, bioluminescence was also not detected (Figure 1D). To further confirm surface GFP-dependent entry, we either added an excess of recombinant GFP to the extracellular medium to compete with the toxin binding or mutated the GFP nanobody portion of the toxin (R30A) (Supplementary Figure 2A) to abolish its recognition of GFP.³¹ Both treatments eliminated bioluminescence. Finally, we showed that reduction of the recombinant toxin with DTT to separate the cargo from the heavy chain prevented entry, but omission of the thrombin cleavage site (Supplementary Figure 2B) did not, suggesting that a covalent link between heavy chain and cargo does not completely impair translocation and cytosolic entry.^{18,24} By comparing the bioluminescence of cells loaded with HiBiT-BoNT-GFPnb to the bioluminescence of purified recombinant NanoLuc, we estimate that our protocol results in delivery of at least 2.4 nM HiBiT cargo to the cytosol of live HEK293T cells (Supplementary Figure 3; see Supporting Information for calculations).

Western blot analysis (Figure 1E) of cellular samples from Figure 1D under non-reducing conditions showed that released HiBiT (55 kDa) was detected only in sample 4, the surface GFP-expressing HEK293T cells treated with HiBiT-BoNT-GFPnb. Control samples did not uptake any toxin, with the exception of sample 7, which contained only full-length toxin due to omission of the thrombin cleavage site.

Collectively, these results show that NanoBiT can be used to read out BoNT-mediated cargo delivery to the cytosol, and that GFP:GFPnb recognition can mediate BoNT surface binding and entry into mammalian cells.

Modularity of BoNT Platform: Different Cargos and Different Binders. To further explore the scope of BoNT-mediated protein delivery, we replaced the GFP nanobody in Figure 1 with a $K_d = 25$ nM nanobody against the extracellular domain of the epidermal growth factor receptor (EGFR)^{33,34} (EGFRnb; Supplementary Figure 2C). EGFR is overexpressed in a number of human cancers (for example, EGFR overexpression is found in at least 50% of triple-negative breast cancers³⁵) and therefore a cell surface marker of great interest for targeted therapies.^{36,37}

We carried out a dose–response experiment, measuring bioluminescence after incubating varying concentrations (0.1–100 nM) of recombinant toxin with HEK293T cells stably expressing cytosolic LgBiT and transiently expressing either surface GFP or EGFR (Figure 2A,B). Note that endogenous EGFR levels are low in HEK293T cells.³⁸ At 100 nM chimeric HiBiT-BoNT-EGFRnb, we detected ~5-fold more uptake of HiBiT into EGFR-expressing cells than into GFP-expressing or untransfected cells, suggesting that the new EGFR nanobody mediates entry via binding to EGFR (Figure 2B). The reverse result was observed with our original HiBiT-BoNT-GFPnb toxin, which gave ~4-fold more bioluminescence when incubated at 10 nM with surface GFP-expressing cells than with EGFR-expressing or untransfected cells (Figure 2A). The lower EC_{50} of the GFPnb toxin compared to the EGFRnb toxin (~3 nM versus >10 nM) correlates with the reported binding affinities of their nanobody domains.^{31,33,34}

Next, we explored alternative cargoes on the BoNT platform, beyond HiBiT. Previous studies using the diphtheria toxin platform have delivered the Ras/Rap1-specific endopeptidase (RRSP), which cuts Ras and Rap1 between Y32 and

D33 of the Switch I region, preventing downstream signaling.^{39,40} RRSP is a therapeutically relevant cargo, as constitutively active variants of Ras (HRAS, KRAS, and NRAS) are among the most common oncogenes.^{41,42} We generated a chimeric toxin consisting of 55 kDa RRSP fused to inactivated BoNT and GFP nanobody (RRSP-BoNT-GFPnb, Supplementary Figure 2D). This material was incubated with HEK293T cells expressing either surface GFP or EGFR. After 24 h, cells were lysed and blotted with anti-Ras antibody to quantify total remaining Ras protein. We observed the greatest Ras decrease (52%) in GFP-expressing HEK293T cells (Figure 2C), which matches the GFP nanobody of our engineered toxin. We also performed the reverse experiment, purifying toxin with EGFRnb in place of GFPnb (Supplementary Figure 2E), and observed the greatest Ras decrease (72%) in EGFR-expressing rather than GFP-expressing HEK293T cells (Figure 2D).

For a third cargo, we utilized the tetanus toxin protease (TTP), which cuts the synaptic vesicle fusion protein VAMP2. We fused 52 kDa TTP to the N-terminal end of our chimeric BoNT and GFPnb to the C-terminal end (Supplementary Figure 2F). The recombinant toxin was incubated with HEK293T cells stably expressing mCherry-VAMP2 reporter, and 24 h after treatment, the cells were lysed and blotted with an antibody that specifically detects cleaved VAMP2.⁴³ After the cells were treated with TTP-BoNT-GFPnb, cleaved VAMP2 was only detected in cells expressing surface GFP and treated with toxin, and not in control cells expressing cytosolic GFP or ER-localized GFP, nor in cells expressing EGFR (Figure 2E). We also prepared a TTP-BoNT-EGFRnb toxin (Supplementary Figure 2G), which was selective for EGFR-expressing cells over GFP-expressing cells, as expected (Figure 2F).

To more rigorously assess the cell-type-specificity of our TTP-BoNT chimeric toxins, we prepared a mixed culture of VAMP2 reporter cells expressing either surface GFP or EGFR (Figure 2G). When the mixed culture was treated with TTP-BoNT-GFPnb, only GFP-positive cells showed staining with antibody against cleaved VAMP2, whereas neighboring GFP-negative cells did not (Figure 2H and Supplementary Figure 4). Conversely, mixed cultures treated with TTP-BoNT-EGFRnb gave cleaved VAMP2 staining on EGFR-positive cells. We also assessed the sensitivity of toxin delivery by calculating the fraction of GFP- or EGFR-positive cells that displayed staining for cleaved VAMP2 (Supplementary Figures 5 and 6). We observed a moderate sensitivity of ~25%, which may be a result of BoNT aggregation (Supplementary Figure 2A–E) that sterically hinders the ability of the translocation domain to dock to the membrane in a productive conformation. Overall, our results demonstrate that BoNT chimeras are able to deliver protein cargo to specific cell subpopulations within heterogeneous cultures.

Simultaneous Delivery of Orthogonal Cargoes to Two Different Cell-Types in Mixed Culture. Encouraged by the cell-type-specificity of the BoNT platform, we explored the possibility of delivering two different cargoes to two different cell-types at the same time, by using orthogonal nanobodies that recognize distinct cell surface markers. We selected TTP as our first cargo and the BoNT/A protease (BTP) as our second cargo. BTP from the BoNT/A serotype cleaves a different presynaptic protein⁴⁵ (SNAP25) than the native protease of our BoNT/X-based platform, which cleaves VAMP2.

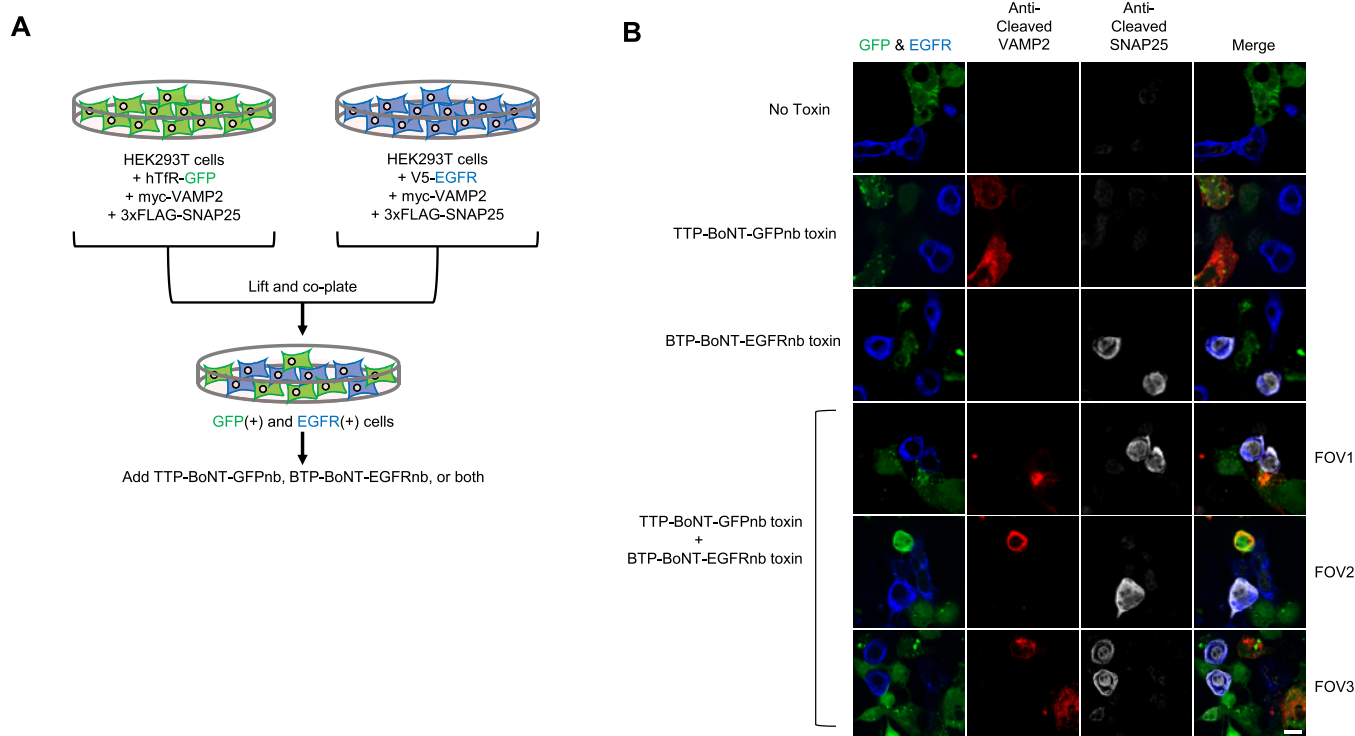


Figure 3. Simultaneous delivery of orthogonal toxins to two different cell-types in an *in vitro* mixed culture system. (A) Experimental design. All HEK293T cells expressed both FLAG-SNAP25 and myc-VAMP2 reporters. (B) Confocal fluorescence imaging of (A). Cells were treated with 50 nM of either TTP-BoNT-GFPnb, BTP-BoNT-EGFRnb, or both for 24 h, and subsequently fixed and stained with anti-cleaved VAMP2, anti-cleaved SNAP25, and anti-EGFR antibodies. In the bottom three rows, showing three different fields of view (FOV), only blue EGFR+ cells show anti-SNAP25 staining, evidence of BTP delivery. Only green GFP+ cells show anti-cleaved VAMP2 staining, evidence of TTP delivery. Scale bar, 10 μm .

We prepared a mixed culture of HEK293T cells expressing either surface GFP or EGFR (Figure 3A). All cells also expressed both myc-VAMP2 reporter and FLAG-SNAP25 reporter (Supplementary Figure 7). We then delivered a mixture of GFP-targeting TTP-BoNT-GFPnb toxin and EGFR-targeting BTP-BoNT-EGFRnb toxin. Cells were fixed and stained with antibodies against cleaved VAMP2 and cleaved SNAP25. GFP-positive cells showed cleaved VAMP2 staining, consistent with delivery of TTP cargo, while EGFR-positive cells showed cleaved SNAP25 staining, consistent with delivery of BTP cargo (Figure 3B and Supplementary Figure 8). At 50 nM of toxin, TTP-BoNT-GFPnb and BTP-BoNT-EGFRnb had $\sim 15\%$ and $\sim 25\%$ sensitivity, respectively, with their target cells 4- and 29-fold more sensitive than non-target cells (Supplementary Figures 9 and 10). These results illustrate the versatility and specificity of the BoNT platform for delivery of different protein cargoes to selective cell populations.

DISCUSSION

In this work, we have shown that BoNT is a versatile platform for cytosolic delivery of protein cargoes in a cell-type-specific manner. We showed that BoNT's receptor binding domain can be replaced by two different nanobody binders, and we demonstrated delivery of four different protein cargoes (HiBiT, RRSP, TTP, BTP) ranging in size from 1.3 to 55 kDa. Our NanoBiT-based live cell assay provides a specific, sensitive, and somewhat quantitative readout of cytosolic cargo delivery, which may also have utility for other protein delivery platforms.

Our study used nanobodies for cell surface marker recognition, while other studies have used receptor binding domains from other toxins^{19,21} or antibody-type binders that require chemical conjugation.^{13,14} Nanobodies are straightforward to introduce to chimeric toxins by genetic fusion and are available for a wide array of cell surface proteins.⁴⁶

The sensitivity of the BoNT platform is comparable to but not superior to those of other toxins. Here we used 10–100 nM of chimeric toxin, which is within the range of concentrations used in other BoNT studies (100 pM–3 nM¹⁹ or 250 nM–1 μM ,²¹ for example). Other toxin delivery platforms have used similar toxin concentrations as well (300 pM–100 nM for anthrax toxin^{13,14} and 10 pM–200 nM for diphtheria toxin^{15–17}). From our imaging studies, we observed that 10–29% of GFP-positive or EGFR-positive cells displayed evidence of matched toxin uptake into the cytosol (evidenced by cleaved VAMP2 or SNAP25 reporter, Supplementary Figures 5 and 9). This mediocre sensitivity may be a consequence of chimeric BoNT's tendency to aggregate, as shown in the non-reducing SDS-PAGE characterization in Supplementary Figure 2A–E. Previous studies have shown that adding a stabilizing peptide to prevent aggregation can increase the efficacy of BoNT-mediated delivery.⁴⁴

Though BoNT has promising capabilities, the platform has significant limitations as well. Several recombinant BoNT fusions that we made in *E. coli* went into inclusion bodies or precipitated after thrombin cleavage, suggesting low solubility. Cargo delivery yield is also limited; though we estimate that ~ 2.4 nM of our smallest cargo, 1.3 kDa HiBiT, could be delivered, larger cargoes are probably delivered in much

smaller quantities. For this reason, enzyme cargoes such as RRSP, TTP, and BTP are attractive because small delivered quantities can exert detectable effects due to catalysis and signal amplification. We attempted but failed to deliver the large non-enzymatic cargoes split-GFP and Gal4 (data not shown).

Several lines of future work could improve the utility and robustness of the BoNT platform. First, a systematic study of the relationships between cargo size, charge, stability, and cytosolic delivery efficiency would aid users in cargo selection and design. Some studies have suggested that the ease with which a cargo can be unfolded and refolded is a major determinant of delivery efficiency.^{18,48} Indeed, BoNT's natural cargo, the 52 kDa metalloprotease that cleaves VAMP2 or SNAP25, is metastable, with a low T_m of 48 °C.⁴⁹ In addition, protein engineering to improve the BoNT chimera's solubility, especially with the aid of computational algorithms such as PROSS,⁵⁰ could also improve the success rate of chimeric BoNTs. Excitingly, recent work²¹ has shown that chimeric BoNT can deliver nucleases (Cas9, Cas13) and recombinases (Cre) into target cells. Together with the programmable cell-type-specificity presented in this work, chimeric BoNT may become a useful platform for intracellular delivery of therapeutic enzymes, such as base editors.⁴⁷

■ ASSOCIATED CONTENT

Data Availability Statement

Plasmids for hTfR-GFP, mCherry-LgBiT-FLAG, mCherry-myc-VAMP2, and V5-EGFR have been deposited at Addgene (accession numbers 199714, 199715, 199716, and 199717, respectively).

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.3c01145>.

Supplementary Figures 1–10; table of plasmids used; table of antibodies used; full experimental details; protein sequences of constructs used (PDF)

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Author Contributions

H.R. and A.Y.T. designed the research and analyzed all the data except where noted. H.R. performed all experiments. B.G.D. provided the anti-cleaved VAMP2 antibody. H.R. and A.Y.T. wrote the paper.

Notes

The authors declare no competing financial interest. The safety protocol for this study was reviewed by Stanford Administrative Panel on Biosafety (APB protocol #3902). All proteins were purified in quantities less than 0.1 mg.

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