

Visualizing the Entry of *Clostridium difficile* Toxin A into Human Colonic Epithelial Cells

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Clostridium difficile is a gram-positive, spore-forming anaerobe that infects the human colon following antibiotic-induced changes to the gut microbiota. Symptoms of *C. difficile* infection (CDI) typically range from uncomplicated diarrhea to life-threatening colitis [1]. CDI is currently the leading cause of antibiotic-associated intestinal disease in the U.S., with health care costs exceeding \$3 billion per year [2, 3]. Outbreaks of multi-drug resistant and hypervirulent strains have led to treatment failures, higher incidence of recurrence and increased mortality rates, creating an urgent need for new therapeutic strategies to treat CDI [4].

The pathogenesis of *C. difficile* is mediated by two large homologous exotoxins, Toxin A (TcdA; 307 kDa) and Toxin B (TcdB; 270 kDa). The toxins are glucosyltransferases that inactivate Rho family-GTPases such as RhoA, Rac1 and Cdc42. Inactivation of these GTPases results in a loss of actin cytoskeletal architecture of cells leading to cell rounding (Fig. 1A and B) and cell death [1]. Cellular toxicity mediated by *C. difficile* toxins therefore depends on receptor binding and subsequent endocytosis into cells. Since these toxins represent excellent targets for therapeutic intervention, understanding the mechanism of toxin entry is a significant priority.

Mammalian cells have several endocytic pathways to internalize nutrients, cargo proteins and to recycle cell surface receptors [5]. These endogenous cellular mechanisms are often exploited by pathogens and virulence factors that hijack the cellular machinery to promote entry into target cells. In the case of *C. difficile* toxins, TcdB has been shown to utilize clathrin-dependent endocytosis to induce cell rounding [6] but the mechanism of TcdA uptake is still unclear. Previous investigations on TcdA uptake implicate both clathrin- and dynamin-dependent and independent pathways, and have relied primarily on inhibitor studies and indirect entry read-outs such as GTPase inactivation and cell rounding [6, 7]. In order to conclusively identify the endocytic pathway(s) exploited by TcdA for intoxication, it was important that we establish an imaging-based system to directly visualize toxin entry in cells. We tried three different strategies for visualizing toxin entry by confocal microscopy. The first strategy involved generating N-terminal AviTagged-TcdA by site-directed mutagenesis, followed by enzymatic biotinylation with an *E. coli* biotin ligase (BirA). The biotinylated toxin was mixed with a ten-fold molar excess of streptavidin-quantum dot (Qdot) conjugates, added to cells, and imaged with an Olympus FV-1000 confocal microscope. Qdot-conjugation allowed us to visualize lower nanomolar quantities of toxin within cells (Fig. 1C and D). However, we did not proceed with this approach due to concerns that the qdots might be altering the entry of toxin, as was previously reported for transferrin and shiga toxin [8]. Our second strategy involved addition of a 3xFLAG tag at the N-terminus of TcdA by site-directed mutagenesis and using anti-FLAG antibodies to detect toxin entry in cells. Our signal-to-noise ratio with this strategy was poor and was not sufficient to perform colocalization studies. Our third strategy involved directly labeling TcdA with reactive Alexa dyes. We were able to label TcdA with thiol-reactive Alexa546, with no loss in toxin function. Caco-2 (human colon adenocarcinoma) cells intoxicated with TcdA-546 were

fixed, stained for clathrin and imaged using a 63x objective (LSM 710 Meta Inverted Confocal Microscope). We did not observe any colocalization of TcdA with clathrin-positive vesicles (Fig. 1D and E) suggesting that TcdA and TcdB might be exploiting different entry pathways to intoxicate cells. Using these labeled toxins as tools, we are now investigating the endogenous endocytic mechanisms of host cells that are hijacked by TcdA to enter and intoxicate their target cells.

References.

- [1] Voth, D. E. and J. D. Ballard, *Clin Microbiol Rev* **18** (2005), p. 247-63.
- [2] Dubberke, E. R. and M. A. Olsen, *Clin Infect Dis* **55 Suppl** (2015), p. S88-92.
- [3] Dubberke, E. R. and A. I. Wertheimer, *Infect Control Hosp Epidemiol* **30** (2009), p. 57-66.
- [4] Kelly, C. P. and J. T. LaMont, *N Engl J Med* **359** (2008), p. 1932-40.
- [5] Doherty, G. J. and H. T. McMahon, *Annu Rev Biochem* **78** (2009), p. 857-902.
- [6] Papatheodorou, P., et al., *PLoS One* **5** (2010), p. e10673.
- [7] Gerhard, R., et al., *J Med Microbiol* **62** (2013), p. 1414-22.
- [8] Tekle, C., et al., *Nano Lett* **8** (2008), p. 1858-65.

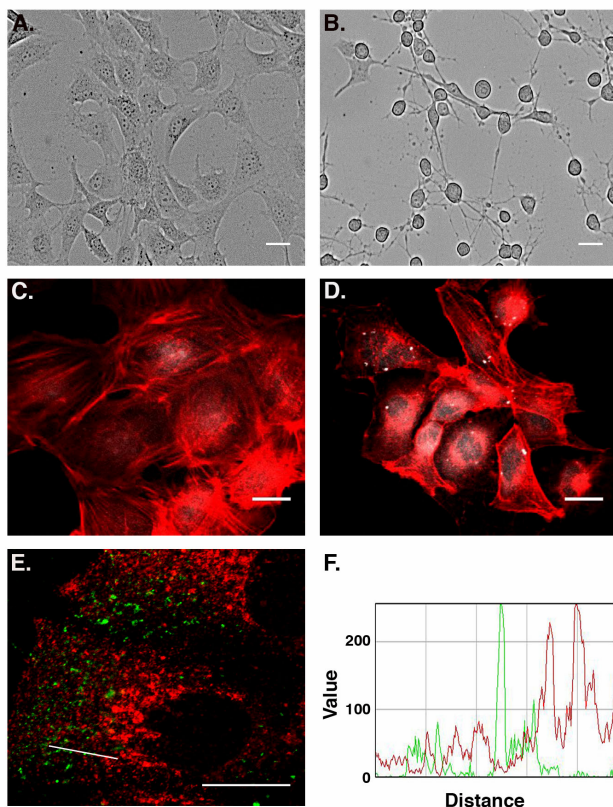


Figure 1. Fluorescently labeled toxin as a tool to visualize and identify TcdA entry mechanism(s) in cells. Cells before (A) and after addition (B) of 10 nM TcdA show the cytopathic ‘cell rounding’ effects induced by toxin. Images were obtained using a Cytation 5 multi-mode plate reader. Scale bar, 100 pixels.

Colonic epithelial cells were treated with Qdot605 only (C) or 1 nM of TcdA-Qdot605 (D; gray puncta), fixed and stained for actin (red). Toxin-treated cells show signs of rounding indicating that the Qdot-conjugation does not affect the function of toxin. Images were obtained using a 60x /1.45 Plan-Apochromat oil lens (Olympus FV-1000). Scale bar, 20 μ m.

E) Colonic epithelial cells treated with 50 nM TcdA-546 (green) for 20 min at 37 °C, fixed and stained for clathrin (red). Images were obtained using a 63x/ 1.40 Plan-Apochromat oil lens (Zeiss LSM 710 Meta Inverted). Scale bar, 20 μ m. F) RGB profile analysis of the tracing shown in (E) reveals a lack of colocalization between TcdA and clathrin.