Bead-based microfluidic toxin sensor integrating evaporative signal amplification†‡

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We have devised a microfluidic platform that incorporates substrate-laden silica beads for sensing the proteolytic activity of botulinum neurotoxin type A (BoNT/A)—one of the most poisonous substances known and a significant biological threat. The sensor relies on toxin-mediated cleavage of a fluorophore-tagged peptide substrate specific for only BoNT/A. Peptide immobilized on beads is recognized and cleaved by the toxin, releasing fluorescent fragments into solution that can be concentrated at an isolated port via evaporation and detected using microscopy. Evaporative concentration in combination with a specific channel geometry provides up to a 3-fold signal amplification in 35 min, allowing for detection of low levels of fluorophore-labeled peptide—a task not easily accomplished using traditional channel designs. Our bead-based microfluidic platform can sense BoNT/A down to 10 pg of toxin per mL buffer solution in 3.5 h and can be adapted to sensing other toxins that operate via enzymatic cleavage of a known substrate.

1 Introduction

Because of its unsurpassed potency, botulinum neurotoxin (BoNT) is recognized by the Centers for Disease Control as a “category A” bioterrorism agent—a high-priority risk to national security upon dissemination.1 Seven antigenic types of BoNT exist (A–G), each of which enzymatically cleaves a select neuronal protein, leading to flaccid paralysis associated with botulism. Accidental or deliberate contamination of vulnerable food supplies, like milk, with BoNT warrants the development of routine, portable screening devices that quickly and confidently identify the toxin.2–3 As a result of the high toxicity (oral lethal dose of 1 µg kg−1 body weight) and dangers posed by BoNT-contaminated samples, a limit of detection (LOD) of 40 pg mL−1 or less has been recommended for botulinum toxin sensors.4

The only approved means of BoNT detection is the mouse bioassay5—an assay that is sensitive down to 1 mouse lethal dose per milliliter (~10 pg), but requires animal sacrifice, trained personnel, special facilities, and lengthy protocols (days). ELISA and other immunoassays have been amended as quick and easy screening tools, but still suffer from insensitivity and cross-reactivity at BoNT concentrations below ~1 ng mL−1.6,7 Other methods of detection such as PCR and mass spectrometry are not amenable for on-site testing due to assay-specific drawbacks such as bulky instrumentation or delicate protocols.8,9 The greatest challenge to date is designing an assay that is both highly sensitive and robust for on-site testing for botulinum toxin in numerous suspect samples.

In response, we present a portable microfluidic platform with multiplexing capabilities for reliable, low-level detection of BoNT type A (BoNT/A) in aqueous solution. Our platform employs a toxin-specific bead-substrate conjugate to screen for BoNT/A enzyme activity wherein BoNT/A cleaves a fluorescent peptide substrate immobilized on the surface of silica beads, releasing the fluorescently-labeled fragments into bulk solution. The sensing mechanism is integrated into a passive microfluidic construct that uses surface tension to manipulate fluid without pumps or tubing. The combination of biorecognition and microfluidic technology allows for detection of low concentrations of BoNT/A with minimal handling. Using this platform, we are able to sense BoNT type A at concentrations up to 4× lower than the recommended LOD in <4 h, thus rivaling the time and experimental constraints of the mouse bioassay. While optimized for BoNT/A, this microfluidic platform could be extended to detecting other enzyme toxins with known substrates including the remaining BoNT serotypes B–G, anthrax, and tetanus toxin. Sensor design considerations and functionality as well as future plans regarding its extension to toxin detection in complex matrices and parallel sensing of other toxic agents will be discussed.

2 Results and discussion

2.1 Sensing component: Bead-peptide conjugate

Our bead-based microfluidic sensor relies on the enzymatic activity inherent to botulinum toxins. BoNT/A is a disulfide-linked, di-chain (heavy and light) 150 kDa polypeptide that specifically cleaves the SNAP-25 protein in the synaptic cleft,
thereby blocking exocytosis of necessary neurotransmitters. As the sequence of the toxin’s in vivo SNAP-25 protein substrate is known, short peptides that mimic the BoNT cleavage site have been designed for enzyme-based assays. The substrate mimic can then be tethered to beads with large surface areas, thus presenting a sufficient amount of substrate to the toxin by adding only microgram quantities of beads to individual microchannels.

2.1.1 Fluorescent SNAP peptide. Previously, we had incorporated a SNAP-25-derived peptide into hydrogels for autonomously sensing BoNT/A proteolytic activity.11 The hydrogels did not possess a sensitivity conducive to low-level toxin detection, presumably due to constraints imposed on the peptide when crosslinked within a gel structure. To mitigate steric constraints and unfavorable peptide conformations, we have focused on tethering only one peptide terminus to silica beads for our current sensor.

The smallest BoNT/A-recognizable sequence is derived from seventeen residues including and surrounding the SNAP-25 cleavage site and has been given in the literature as: SNKTRIDAEANQRATKML.12 This core 17mer was modified for our particular sensing application. We synthesized a 24mer containing an N-terminal fluorescein (flu) for detection and a C-terminal cysteine for thiol-selective conjugation to maleimide-functionalized silica beads. Similar fluorogenic substrates have been used for sensing BoNT/A holotoxin or light chain and for exploring the efficacy of BoNT inhibitors.13–17 The resulting sequence—fluGGGSNRTRIDAEANQRATRKLGGGC—will herein be referred to as “flu-SNAP.” Other complementary modifications to the peptide, namely K3R, K15R, M16Nle, and glycine spacers (GGG) have been described in detail previously.11 Flu-SNAP was shown to be a suitable substrate for the toxin using HPLC with peak area ratios indicating 25% cleavage after 5.5 h and 48% cleavage after 8.25 h (Fig. 1).

2.1.2 Bead-flu-SNAP conjugation. Generally grouped together as “beads,” nanoparticles, microparticles, and microspheres are employed in assays for increased surface area as well as ease of manipulation and versatile chemical modification. The tethering of biomolecules to beads has been widely adapted to sensing applications with a variety of outputs.18–23 Most relevantly, Bagramyan et al. employed antibody-laden beads to capture BoNT/A and measure enzyme activity with a FRET substrate23 and Moorthy et al. used avidin-modified agarose beads as the sensing element within a comprehensive, microfluidic-based ELISA for BoNT/A.19

To simplify our platform in relation to previously developed assays, we focused solely on the enzymatic activity of the toxin without the added complication of immuno-based methods. The BoNT/A-recognizable flu-SNAP was directly conjugated to maleimide-functionalized silica beads (35–70 μm) via free C-terminal thiols to form a stable thioether bond (Fig. 2a). Immobilization, which consisted of incubation with peptide and multiple wash steps, took a total of 4–6 h. Aliquots (0.5 mL) of the bead suspension (≈150 mg mL−1) were then introduced to PDMS microchannel reservoirs already containing 4 μL either toxin or control solutions.

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**Fig. 1** HPLC reveals the ability of 2 μg mL−1 BoNT/A light chain to cleave 48% of the flu-SNAP substrate (100 μM) over the course of 8 h as determined using the ratio of fragment (B) to total (intact + fragment, A + B) peptide peak areas. Chromatograms timepoints are, in order: 0, 185, 330, 495 min.

**Fig. 2** Diffusion valve microchannel and peptide-bead conjugation scheme: (a) Peptide (flu-SNAP) was bound to maleimide-silica beads and loaded into the large “reservoir” (3.4 mm²) containing the toxin sample. After incubation, the channel is placed in a non-humidified environment, generating evaporative flow from the large reservoir to the small detection port (diameter, 750 μm) for fluorescent imaging. (b) Bright-field image of arrayed diffusion valve microchannels. (c) Bright-field image of channel filled with beads shows maximized bead coverage in reservoir. (d) Representative fluorescent image of channel after incubation with toxin and evaporation. The diffusion valve completely isolates fluorescent analyte in the detection port, preventing diffusion “upstream” into the channel and amplifying the output signal. Scale bars (c, d): 500 μm.
Silica beads proved to be very “sticky” and difficult to remove the nonspecifically adsorbed peptide. An abundance of adsorbed peptide led to higher control (blank) signals as peptide desorbed over the course of the assay. To reduce this effect, various wash steps and subsequent solution removal techniques were tested. Washed beads were exposed to control buffer only (30 mM HEPES, pH 7.4 with 1 mg mL⁻¹ BSA) for 3 h at 30 °C, solution removed, and fluorescence measurements taken off-chip using a fluorospectrometer. We found that the most effective wash routine—adopted from Schmidt et al.⁵—was 50 mM Tris/0.1% Tween-20, pH 8.0 with 5 mM EDTA and 40 mM 2-mercaptoethanol followed by 5 min sonication and brief washes with 1× PBS, pH 7.4. This routine provided nearly a 3-fold decrease in control buffer fluorescence as compared to beads washed with only deionized water and 1× PBS (data not shown). No difference was found in the method of solution removal (centrifugation followed by supernatant aspiration or centrifugation in cellulose acetate spin cups) with regard to decreased blank signal. Despite a decrease in buffer fluorescence, it is clear that peptide continues to desorb from the silica surface over time and raise the background signal. More efficient washing may be achieved from the use of magnetic beads that can be effectively removed from the solution by applying a magnetic field or from using “bio-inert” beads that are, for example, PEG-based and more resistant to protein adsorption than silica.

2.2 Microchannel design

A BoNT inhibitor-screening assay was recently formatted for the microscale by using a FRET peptide substrate within microchannels,¹⁴ however, the device was complex, requiring extensive fluidic components for fluid manipulation as well as multiple inlet and outlet ports for reagent addition/removal. Toward simplicity for the end-user and straightforward field-testing of suspect samples, our entire proteolysis assay (incubation, analyte concentration, and fluorescence detection) takes place in a single channel. The assay does not necessitate any hazardous sample transfer between assay steps and requires only a pipet to add sample and bead suspension.

Several features were integrated into the device design to leverage useful aspects of microchannels (Fig. 2). First, careful designing of the loading port provided increased interaction of substrate-modified beads and the toxin solution for optimized reaction rate and efficiency. Next, to enable an integrated readout, flow was then generated in the channel to transport the solution in the reservoir to an area optically isolated from the bound fluorophore. Finally, a passive evaporation-based amplification system was designed to effectively trap the fluorescent analyte of interest in a confined “detection port” of set volume, which provides an amplification step for a more sensitive readout. The uncomplicated device design lends itself to scale-up for high-throughput sensing (Fig. 2b) with potential automation by means of robotic pipetters and/or on-chip fluorescence detectors.²⁴₂⁵

2.2.1 Reaction optimization. In addition to increasing readout efficiency, the microchannel can be tailored for better reaction efficiency. To cleave as much fluorophore as possible during a set incubation time, the toxin must be in close proximity to the beads and the diffusion length reduced. This was achieved by removing the channel roof above the bead reservoir, providing access for pipetting the bead suspension directly into the bulk toxin solution. The large, open elliptical reservoir minimizes diffusion distances and maximizes the number of beads that can be deposited (Fig. 2c).

2.2.2 Integrated readout. Botulinum neurotoxin cleaves fluorophore-labeled peptide off of the silica beads, thereby increasing the fluorescence of the solution in the reservoir. Integration of the readout directly into the device is achieved by flowing the fluorescent solution surrounding the beads to a clean area devoid of bound peptide and beads. Changes in surface tension induced by evaporation generate flow from the large reservoir to the small detection port,²⁶⁻²⁷ which consequently transports fluorophore to the detection port. This enables straightforward imaging using a microscope or scanner.

The flow rate $Q$ is given by eqn (1), where $D_{H_2O}$ is the diffusion coefficient of water in air, $RH$ is the relative humidity of the atmosphere (~40%), $P_{H_2O}$ is the saturation concentration of water vapor (23 g m⁻³ at 25 °C), $\rho_{H_2O}$ is the density of water, and $R$ is the radius of the detection port:

$$Q = \frac{2D_{H_2O}(1 - RH)\rho_{H_2O}^*}{\rho_{H_2O}/R}$$  \hspace{1cm} (1)$$

The parameters included in eqn (1) can be readily tuned to obtain a flow rate desirable for a particular application by, for example, altering channel design (port radius) or assay conditions (temperature, humidity). Evaporative flow is advantageous for our sensor as it is fully autonomous and naturally provides flow rates small enough to prevent beads from being extracted from the reservoir.

2.2.3 Readout amplification. Evaporation at the detection port leads to an increase in fluorescent peptide concentration, which provides for a built-in amplification mechanism. A large droplet of water (10 μL) is placed over the sample reservoir to provide continual flow as solvent evaporates from the detection port. We show that using a traditional straight channel where the output port diameter is roughly equal to the channel width, the signal for dextran-conjugated Alexa 488 (10 kDa) is amplified 1.7× after 45 min of flow (Fig. 3).

The rate of amplification is theoretically proportional to the flow rate from eqn (1). For an initial concentration in the detection port $C_i$, an initial concentration in the reservoir port $C_i^*$, an evaporation driven flow rate $Q$, and a detection port volume $V$, the amplification over time $A(t)$ is given by eqn (2):

$$A(t) = 1 + \frac{C_i Q}{C_i^* V}$$  \hspace{1cm} (2)$$

Despite the amplification enabled by the use of evaporation, diffusion of the analyte “upstream” into the channel from the detection port can cause significant dilution and reduce amplification. This phenomenon occurs particularly for small fluorophores that can compete with the small convective flow induced by evaporation. To counter this, a passive “diffusion valve”—a constricted channel (80 μm × 80 μm, width × height) connecting two large ports—was designed to trap the fluorescent peptide fragments at the detection port (Fig. 2).
Evaporative concentration enables signal amplification of dextran-conjugated Alexa 488 (10 kDa). Traditional straight channels experienced only 1.7× signal amplification in 45 min (solid red line) whereas “diffusion valve” microchannels provided nearly 3× amplification in the same time period (solid black line). Theoretical amplification over time based on experimental diffusion valve conditions was determined using eqn (1) and (2) (dotted black line).

As the flow rate of the fluid is set by the magnitude of evaporation, the velocity can be altered by varying the cross-sectional area of the constricted channel. For a channel with a small cross-sectional area, high velocity prevents diffusion from transporting elements upstream. The constricted diffusion valve (80 μm width) thus serves to trap the fluorophore in a small volume (detection port), effectively isolating and amplifying the fluorescent signal (Fig. 2d). Increased velocity and concentration provided by the diffusion valve has been further illustrated for the two different channel designs and is illustrated using both models and representative fluorescent images in Fig. 4. Utilizing this approach for a microchannel design, we show that amplification of 3–4× is realizable in under one hour (dotted black line, Fig. 3). For our sensor, a set 35 min evaporation period was used to limit assay time while retaining sufficient evaporative concentration. With longer evaporation times and continual reservoir replenishing, however, the theoretical amplification maximum ($A_{\text{max}}$) for our given channel geometry would be ~20, as given by eqn (3):

$$A_{\text{max}} = \frac{V_{\text{sample}}}{V_{\text{output port}}}$$

### 2.3 Sensing BoNT/A light chain and holotoxin

The bead-based microfluidic sensor was tested with a range of toxin concentrations above and below the recommended LOD of 40 pg mL$^{-1}$. Initial testing took place with the purified 50 kDa BoNT type A light chain (ALC), which is known to be a nontoxic catalytic substitute for the toxin. Each channel received ALC at concentrations ranging over six orders of magnitude (20 pg mL$^{-1}$–20 μg mL$^{-1}$) followed by 0.5 μL of bead suspension. After a 3 h incubation at 37°C and 35 min of evaporative concentration, fluorescent images were taken at the detection ports and the intensities quantified and normalized to an average control (blank) signal (30 mM HEPES buffer containing 1 mg mL$^{-1}$ BSA). Fluorescence output increased with ALC concentration and the resulting curve suggests cleavage of flu-SNAP was dependent on both the amount of toxin present in the sample and on flu-SNAP concentration (substrate-limiting conditions) (Fig. 5).

BoNT/A light chain data were “fit” to a nonlinear representative Michaelis–Menten formula, \( \frac{Ax}{(B + x)} + C \) to reveal a general trend in fluorescence output (gray curve). The smallest output detectable with 95% confidence ($x_L$) was determined from experimental control data \((n = 10)\) using eqn (4), where $\bar{x}_b$ and $s_b$ are the respective mean and standard deviation of the blank measurements and $k$ is the confidence level (0.95):

$$x_L = \bar{x}_b + ks_b$$

The $x_L$ for sensing the ALC is plotted as a dotted grey line and represents the value at which sensor output cannot be confidently discerned from the corresponding control signal. As such, the 20 pg mL$^{-1}$ (0.4 pM) ALC data point, while displaying a greater average signal than the control, falls below this line and

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**Fig. 3** Evaporative concentration enables signal amplification of dextran-conjugated Alexa 488 (10 kDa). Traditional straight channels experienced only 1.7× signal amplification in 45 min (solid red line) whereas “diffusion valve” microchannels provided nearly 3× amplification in the same time period (solid black line). Theoretical amplification over time based on experimental diffusion valve conditions was determined using eqn (1) and (2) (dotted black line).

**Fig. 4** COMSOL models of fluid velocity and concentrating effects after 15 min of evaporation for (a) diffusion valve channels (80 μm width) and (b) traditional straight channels (700 μm width). Fluid velocity in the constricted diffusion valve reaches 20 times that of the straight channel, resulting in greater concentrating effects and signal amplification. Representative fluorescent images are shown for each channel (far right). Scale bar: 175 μm.
Fig. 5 Microchannel detection port fluorescence resulting from both BoNT/A light chain (solid squares) and holotoxin (solid circles) cleavage of immobilized flu-SNAP from silica beads. Amplified fluorescence signals were normalized to respective control buffers without toxin. Data were fit to nonlinear trendlines (solid curves) and curve variation between ALC and the holotoxin probably reflects differences in enzyme responsiveness to the immobilized peptide. The smallest detectable measurement \( \chi_i \) for the sensor with respect to both toxin controls was determined using eqn (4) and is plotted as dotted lines (blue—BoNT/A holotoxin; gray—ALC).

Therefore is below the limit of detection. It is estimated from the actual data, however, that ALC concentrations above ~100 pg mL\(^{-1}\) should be detectable.

It has been generally accepted that the toxin has higher activity at 37 °C, yet we note lower activity at 37 °C as compared to 30 °C when tested with ng mL\(^{-1}\) concentrations of ALC \((n_{\text{total}} = 8)\) (Fig. 6). It was found that the toxin sensor performed better at 30 °C, averaging a 1.8-fold signal increase for both 2 and 200 ng mL\(^{-1}\) ALC. This could be explained by the small amount of precipitation noticed with reduced BoNT/A at temperatures above 35 °C. Despite the increased fluorescent readout at 30 °C, the signals were not significant compared to those at 37 °C for 200 ng mL\(^{-1}\) \((P = 0.10)\) or 2 ng mL\(^{-1}\) \((P = 0.14)\). Nonetheless, to prevent any loss of activity from temperature-induced enzyme precipitation and to improve assay readout based on experimental observations, the temperature was kept at 30.5 ± 0.5 °C for testing with the live BoNT/A holotoxin.

Having verified sensor function with ALC, flu-SNAP beads were then exposed to the live 150 kDa BoNT/A holotoxin at concentrations spanning five orders of magnitude (10 pg mL\(^{-1}\)–0.67 μg mL\(^{-1}\)). After a 3 h incubation at 30 °C, fluorescence values were quantified and normalized to an appropriate control (blank) signal (30 mM HEPES buffer containing 1 mg mL\(^{-1}\) BSA and 1.3 mM DTT). Fluorescence output increased with BoNT/A concentration and similarly suggests cleavage of flu-SNAP was dependent on both the amount of toxin present in the sample and on the amount of immobilized flu-SNAP (Fig. 5). Data were fit to a representative nonlinear Michaelis-Menten formula mentioned previously to show the general trend in fluorescence output (blue curve). The smallest detectable value \((\chi_i)\) was determined from experimental BoNT/A control data \(n = 12\) using eqn (4) and is plotted as a dotted blue line. All BoNT/A concentrations produced fluorescent signals significantly above the average control value and above the calculated \(\chi_i\), thus demonstrating the ability of the bead-based sensor to detect down to 10 pg mL\(^{-1}\) (67 fm) BoNT/A holotoxin.

It follows, then, that all signals are above the LOD and it may be possible to sense lower BoNT/A concentrations, especially upon further reduction of background signal \(i.e.,\) controlling flu-SNAP desorption from beads. Actual sensor data for BoNT/A light chain and holotoxin are provided in Tables S1 and S2.

The offset curves for the respective ALC and BoNT/A data can be attributed to differences in enzyme responsiveness to the immobilized flu-SNAP as the holotoxin and its light chain possess discrete enzymatic properties. However, the inability to exactly calculate the flu-SNAP concentration in each microfluidic reservoir precludes any concrete kinetic interpretation of these data. Error bars along the y-axis representing standard error \((s/\sqrt{n})\) for a given sample set ranged from 9–14% for ALC and 6–13% for BoNT/A and followed no trend based on toxin concentration. Intra- and inter-assay variability likely results from the inability to precisely deliver the same amount of peptide-laden beads to individual reservoirs as silica beads are very dense and settle within seconds, making prolonged bead suspension in buffer (>1 min) impossible. As such, variability could be lessened by the use of either lighter beads to achieve a more stable, homogeneous suspension or micro stir bars that have been shown to maintain cell suspension consistency, for example.

2.4 Cross-reactivity and interfering agents

Susceptibility of an assay to extraneous sample components is an important aspect to consider when developing a multiplexed sensor capable of simultaneously detecting more than one toxic agent. It is therefore critical that the BoNT/A sensor does not cross-react with any of the other BoNT serotypes. BoNT type B (BoNT/B) is also toxic to humans, but does not have a cleavage site within SNAP-25 and therefore should not recognize the flu-SNAP substrate. BoNT/B was tested with our microfluidic bead-based sensor to determine the level of cross-reactivity, if any. At a concentration of 10 ng mL\(^{-1}\), BoNT/B did not produce...
a significant fluorescent signal as compared to control buffer with 1.3 mM DTT ($P = 0.18$) (Fig. 7). The same concentration of BoNT/A, however, produced a signal almost twice that of the control with <7% error ($*P < 0.001$). BoNT/B at 1 $\mu$g mL$^{-1}$ similarly showed no activity in the presence of flu-SNAP beads (Table S3$^\dagger$). It is then reasonable to assume that the sensor is specific for only type A BoNT and is insensitive to type B enzyme activity. Because of the versatility in our assay design and negligible cross-reactivity, it would be possible to also tether the BoNT/B peptide substrate to the beads and sense both serotypes in parallel.

It is also noteworthy that DTT (dithiothreitol) is not an interfering agent in assay performance as compared to control buffer without DTT ($P = 0.4$). DTT is a necessary component in activating BoNT/A via reduction of the disulfide bond adjoining the heavy and light chains and remains in the buffer during the entire assay. It has been found to suppress BoNT hydrolysis at high concentrations$^{28}$ and can interfere in certain assays that rely on maintenance of disulfide$^{38}$ or metal–thiol bonds.$^{50}$ For these reasons, DTT was a concern when optimizing assay conditions, but did not appear to adversely affect the assay at low concentrations (1.3 mM).

Lastly, we preliminarily addressed the possibility of using the sensor for milk samples containing BoNT/A since the milk supply is a tangible target for botulinum toxin dissemination.$^3$ Certain milk proteins, however, are known to autofluoresce$^{41}$ and could lead to higher background signals and/or false-positives. We, too, have seen a large degree of autofluorescence in the visible range and were unable to discern cleaved flu-SNAP signal from a control milk sample (data not shown). Future work will center on the use of other fluorophores such as IR dyes that require longer wavelength excitations, thus making our bead-based microfluidic platform available for testing suspect liquid food and drink samples.

### 3 Conclusion

As an alternative to the standard mouse bioassay, a bead-based microfluidic sensor for botulinum neurotoxin type A with low-level detection capabilities and negligible cross-reactivity has been realized via the coupling of biochemistry and fluidics. Enzyme-substrate biorecognition provided for selectivity for type A BoNT, while an integrated “diffusion valve” isolated and amplified the resulting fluorescent signal to facilitate detection of live BoNT/A down to 10 pg mL$^{-1}$ buffer solution (~67 IM) in 3.5 h. Ease of use, built-in evaporative signal amplification, and potential scalability could allow for high-throughput detection of trace levels of BoNT/A or other enzyme toxins with minimal sample handling or manipulation.

### 4 Experimental

#### 4.1 Reagents

Trifluoroacetic acid (TFA) was purchased from Aldrich Chemicals. Tween-20 was purchased from Acros Organics. Dextran-conjugated Alexa 488 (10 kDa) was purchased from Invitrogen. Cellulose acetate spin cups were purchased from Pierce. All other reagents were obtained from Fisher. Botulinum neurotoxin serotypes A (holotoxin and light chain) and B (holotoxin) were generously provided by the Johnson laboratory at University of Wisconsin-Madison. Deionized ultra-filtered (DIUF) water was used for all solutions. A NanoDrop 3300 (Thermo Scientific) fluorospectrometer was used for off-chip fluorescence measurements. All bright field and fluorescent images were taken on an Olympus IX70 microscope with Metamorph software and processed using ImageJ (NIH).

#### 4.2 Peptide synthesis

Flu-SNAP was synthesized on a Symphony instrument (Protein Technologies, Inc.) in the UW-Madison Peptide Synthesis Facility using standard solid phase synthesis methods on Rink Amide resin (Novabiochem). The peptide was C-terminus amidated and 5-carboxyfluorescein (flu) (Novabiochem) was added to the N-terminus. Peptide was deprotection for 4 h using a TFA cocktail containing 5% (v/v) thioanisole (Acros Organics) and 2.5% (v/v) ethanediol (Alfa Aesar). Flu-peptide was purified using reverse phase RP-HPLC on a 5 $\mu$m preparative scale C-18 column using a 15–50% gradient of 0.08% TFA in ACN against 0.1% TFA. Lyophilized peptide was stored at $-20$ °C until use.

#### 4.3 HPLC of flu-SNAP substrate with BoNT/A light chain

RP-HPLC was used to verify toxin recognition of the synthetic 2.8 kDa SNAP peptide substrate containing an N-terminus flu. A Prominence HPLC (LC-20AT) with a PDA detector (SPD-M20A) and C-18 column (5 $\mu$m) were used (Shimadzu Corp.). Mobile phase consisted of 0.1% TFA in HPLC grade acetonitrile (Fisher). BoNT/A light chain (2 $\mu$g mL$^{-1}$) was added to 100 $\mu$L flu-SNAP dissolved in 30 mM HEPES with 1 mg mL$^{-1}$ BSA and incubated at 37°C. Aliquots (20 $\mu$L) were removed at...
designated timepoints and the pH was lowered to 2 with a 1% TFA solution. Chromatograms were generated using Shimadzu EZStart software (v. 7.4SP1).

4.4 Bead conjugation

Flu-SNAP (1.7 mg) was added to 1 mg of 3-(maleimido)propyl-functionalized silica beads (200–400 mesh, 0.7 mmol g⁻¹ loading; Sigma) in 1× PBS, pH 7.4 for 2–4 h at RT. Beads were washed with 50 mM Tris/0.1% TWEEN-20, pH 8.0 with 5 mM EDTA and 40 mM 2-mercaptopethanol, sonicated for 5 min, and finally washed three times with 1× PBS, pH 7.4. Cellulose acetate spin cups were often used to remove wash solutions. Beads were resuspended in DIUF at a concentration of 150 mg mL⁻¹ and stored at −20 °C until use.

4.5 Device fabrication

Microchannels were fabricated using standard soft lithography and PDMS (poly(dimethylsiloxane), Sylgard 184, Dow Corning). Molds (masters) were created by coating SU-8 100 photoresist (Microchem) on a silicon wafer. The first layer (80 μm) defined the diffusion valve channel and elliptical bead reservoir and the second layer (250 μm) defined the ports. The master-making process is described in detail elsewhere.32 PDMS was poured on the resulting master, covered with transparency film, and weighted with 20 lb to ensure complete port formation without thin films covering them. This stack was baked on a hot plate at 80 °C for 3 h after which the PDMS was peeled off the master and placed on a glass microscope slide.

4.6 Sensor testing with BoNT/A holotoxin and light chain

4.6.1 Sample preparation. Concentrated BoNT/A and BoNT/B were reduced with 50 mM DTT at RT for 45 min. Stock solutions (0.1 mg mL⁻¹) were diluted in 30 mM HEPES with 1 mg mL⁻¹ BSA, pH 7.4 keeping DTT concentration at 1.3 mM. BoNT/A light chain (ALC) samples were diluted from a frozen 2 mg mL⁻¹ stock solution in HEPES/BSA. The control buffer for all BoNT/A and BoNT/B experimentation was HEPES/BSA with 1.3 mM DTT. The control buffer for all ALC experimentation was HEPES/BSA. Note: All work with BoNT holotoxin must take place in certified laboratories with proper precautions.

4.6.2 Assay conditions. A 4 μL aliquot of toxin sample was added to each diffusion valve microchannel followed by 0.5 μL of flu-SNAP bead suspension. Up to 24 channels were incubated simultaneously in a humidified environment at either 30 or 37 °C for 3 h. After incubation, 10 μL droplets of DIUF were carefully placed on the reservoir ports and evaporation was allowed to take place in a non-humidified environment for 35 min. Detection ports were imaged via fluorescence microscopy.

4.7 Data processing

To remove experimental error from all data sets, we used the sample median as a robust estimator.33 A sample median (T) was found for all intensity values within a particular data set from which a median of all absolute deviations (MAD) was determined as a scale estimator (S). Individual datum z scores (z) were used to remove outliers (eqn (5)): data with a z-score above an arbitrary 2.5 cutoff were chosen to be excluded from the data set as the probability that z > 2.5 is very small for a data set with an otherwise normal distribution.

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z = \frac{x - T}{S}
\]

Averages and standard deviation (s) were calculated for each data set and then normalized to a control (blank) signal. LOD was determined using the eqn (4) at 95% confidence. Standard error (s/√n) was plotted as y-axis error bars to reflect population size. Actual values are included in the ESI.4 Student’s t-test (one- or two-tailed, depending on experimental hypotheses) was used to determine the significance of signals denoted with “*” or “NS.”

4.8 COMSOL models

Diffusion valve and straight microchannels were modeled using COMSOL Multiphysics (v. 3.3). Channel geometry for diffusion valve model: port (h, 400 μm; r, 375 μm), diffusion valve (3 mm × 80 μm × 80 μm, l × w × h). Channel geometry for straight channel model: port (h, 400 μm; r, 375 μm), channel (3 mm × 700 μm × 200 μm, l × w × h). Physical constraints: diffusion coefficient (150 μm² s⁻¹), initial concentration (in evaporation port, 1; in rest of device, 0).

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