Electronic Supplementary Information - ESI

Rapid and highly sensitive luciferase reporter assay for the automated detection of botulinum toxin in the centrifugal microfluidic LabDisk platform

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S1 Optimization of microfluidic protocol

Bead separation
The separation of the particles after incubation is performed using a combination of centrifugal sedimentation and siphon-based valving. Using a volume triggered siphon rather than a capillary-force triggered siphon has the advantage of being more robust and unaffected by the polarity of the sample. After incubation in the BoNT LabDisk (**Fig. S1a**) it is crucial to have all the beads separated from the liquid phase since transferred beads would cause a high luminescence background noise in the detection chamber (**Fig. S1b**). With a rough calculation the centrifugal protocol has be adjusted to ensure complete separation:
Centrifugal force to separate the beads is applied (5 seconds at 30 Hz) before the siphon is primed at 40 Hz (**Fig. S1c**).

The drag force acting on a single spherical bead is estimated using Stokes' law for spherical particles under laminar conditions:

$$F_d = 3\pi\mu D v \tag{1}$$

with the velocity v being measured as 0.8 m/s (100 µl passing through a 200 µm square channel in 3.0 seconds at 40 Hz), the particle diameter D of 45 – 165 µm, and the dynamic viscosity µ estimated with 1 – 3 kNm^{-2} depending on the sample/buffer matrix . The counteracting force is the centrifugal force partially compensated with the lift force:

$$F_z = ma = \Delta\rho \frac{\pi D^3}{6} r(2\pi f)^2 \tag{2}$$

with m being the compensated mass derived from the difference in density $\Delta\rho$ (Sepharose in aqueous buffer estimated with 50 kgm^{-3}), r being the radial position of the bead in the siphon (ranging from 44 – 40 mm) and f being the priming frequency of 40 Hz. In any assumed case $F_d > F_z$ which means that once the siphon is activated, beads in the siphon would be transferred with the liquid flow. Therefore, sufficient sedimentation time has to be applied before the priming buffer is washed into the incubation chamber. Sedimented beads will not be affected by the flow in the siphon and will stay in the incubation chamber. The sedimentation velocity according to Stoke's law of settling can be expressed with

$$\frac{dr}{dt} = \frac{D^2\Delta\rho}{18\mu}(2\pi f)^2 r \tag{3}$$

which leads to the sedimentation time t with r_0 and r_1 being the redial positions of the inner- and outermost level of the liquid in the incubation chamber (40 and 45 mm):

$$\int_0^t dt = \frac{18\mu}{D^2\Delta\rho(2\pi f)^2} \cdot \int_{r_0}^{r_1} \frac{dr}{r} \quad (4)$$

and:

$$t = \frac{18\mu}{D^2\Delta\rho(2\pi f)^2} ln\frac{r_1}{r_0} \quad (5)$$

Even for the smallest particles and the highest assumed viscosity the sedimentation time is < 2 seconds which means that 5 seconds at 30 Hz ensures sufficient separation of the beads for the subsequent priming at 40 Hz.

The estimation of the sedimentation time is consistent with our experimental data. For all blank samples (functionalized beads but no BoNT in buffer), the normal background noise of the detector cannot be distinguished from the background in the luminescence chamber. Furthermore, in microscopic examination no beads were found in the detection chamber or in the siphon.

Mixing
Magnetic microbeads (HaloLink G931A) can be attracted by a magnet that is integrated in the LabDisk Player (for 5 seconds with the magnet placed in the center of the incubation chamber below the disk). Alternating centrifugation (2 seconds at 10 Hz with 5 Hz/s acceleration) and attraction of the beads by the magnet ensures constant mixing. Non-magnetic beads (G1914, Promega) were incubated at 0.04 Hz with the disk reader on its side (or with a 45 ° tilted disk reader) to ensure proper mixing with the beads and sample.

Reagent release
Liquid reagents were introduced into the storage chambers by the operator just before beginning the assay. The size of the capillaries and the radial positions of the chambers define the spinning frequency for the reagent release. For long term pre-storage of liquid reagents, the luminescence substrate and the priming buffer can be stored in foil pouches, so called miniature stick-packs.[1] The stick-packs burst at a defined spinning frequency of the LabDisk and release the liquid. Stick-packs with priming buffer have been tested (burst frequency of 50 Hz, ~100 kPa fluidic pressure). However, the tests in this study correspond to reagent release without stick-packs.

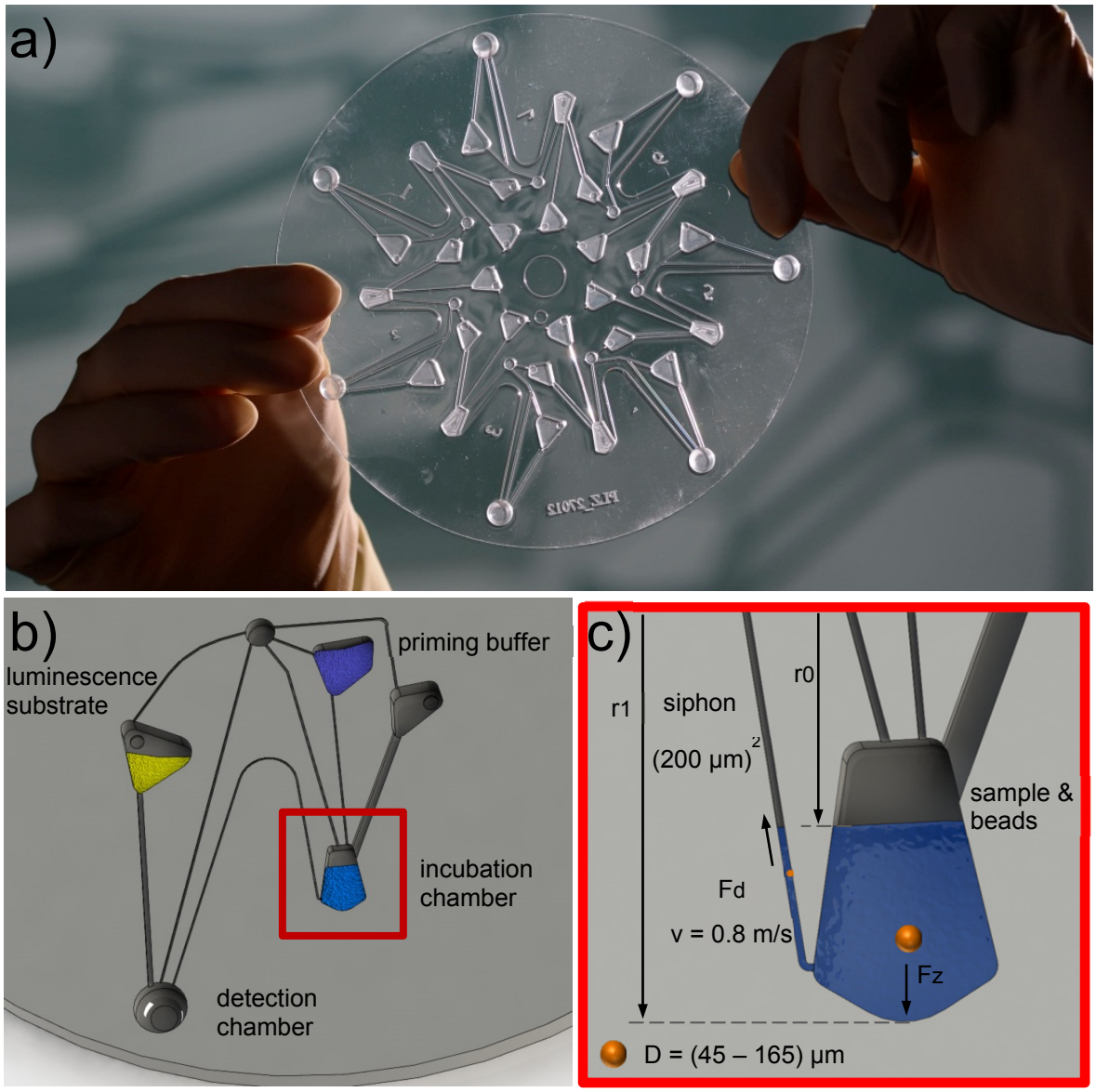

Fig. S1: BoNT LabDisk. a) LabDisk comprising seven individual tests; b) design of an individual test. Micro-beads, buffer and sample are incubated in the incubation chamber; and, c) scheme of beads in the incubation chamber and siphon outlet.

S2 Microwell plate assay (manually performed luciferase assay)[2]

7.5 µl HaloLink Magnetic Beads (G931A, Promega) functionalized with HA-2SL were dispensed into wells of a white NBS 96 well microplate (3990, Corning B.V. Life Sciences, Amsterdam, The Netherlands). BoNT/LC was serially diluted in LCA Hydrolysis Buffer (50 mM HEPES, 1 mg/ml BSA or 0.05% TWEEN 20, pH 7.4, titrated with K HEPES salt) and 4.74 µl samples were transferred to wells containing the functionalized beads. The plates were covered and incubated at room temperature, with agitation on a HydroFlex microplate washer (Tecan Group Ltd, Männedorf, Switzerland). PBS Buffer (50 µl, 150 mM NaCl, 10 mM Na2HPO4, pH 7.4) was dispensed into the bead/sample mixture. For each well, the functionalized magnetic beads were collected with the aid of a magnet, and 50 µl supernatant (50 µl) was transferred to a fresh well of the micro plate. Bioluminescence was detected in a PHERAstar Plus microplate reader (BMG LABTECH GmbH, Ortenberg, Germany) after addition of 25 µl Bright-Glo Luciferase Assay System E2620 (Promega).

S3 Analytical performance

The limit of detection (LOD) and analytical sensitivity were calculated using the blank values of the disks and the lowest concentration of the detected samples (**Table S.3.1**).

Table S3.1 indicating the average and standard deviation of four repeats of a blank and two dilutions

	Average [a.u.]	Standard deviation [a.u.]
Blanks (BoNT/LC disk)	7.5	2.2
BoNT/LC 9 pM	18.6	5.7
Blanks (BoNT disk)	5.9	0.9
BoNT 9 pM	6.9	1.9
Blanks (BoNT complex disk)	5.8	4.2
BoNT complex 9 pM	9.8	2.3

The LOD was calculated using the standard analytical formula: Signal corresponding to the LOD = Average signal of the blank from all the repeats + 3 (standard deviation of blank). The concentration corresponding to this signal corresponds to the LOD.

The analytical sensitivity (AS) was calculated using the standard analytical formula: Signal corresponding to the AS = Average signal of the lowest concentration + (3 standard deviation). The concentration corresponding to this signal corresponds to the AS.

The LabDisk platform is intended for point-of-need applications, where analysis time should be < 45 minutes in order to compete with the commercially available products. This was the reason that we chose 20 minutes incubation in order to have the desired sensitivity for the intended point-of-need application. On the other hand, the longer incubation time (several hours) based developed assay, leading to low LOD and EC50, are intended for standard laboratory-based analysis (**Table S3.2**).[2]

Table S3.2 Analytical performance of the standard laboratory-based luciferase reporter assay with longer incubation times.

BoNT type	**Matrix**	**Incubation time [h]**	**EC50 [pM]**	**LOD [pM]**
BoNT/A1	Buffer	4	13.8	0.1
BoNT/A1	Buffer	18	0.89	0.04
BoNT/A1	Citrate plasma	21	30.5	2.0

S4 References

1 T. van Oordt, Y. Barb, J. Smetana, R. Zengerle and F. von Stetten, *Lab Chip,* 2013, vol. *13* (15), pp. 2888-2892.

2 G. Stevens, D. A. Silver, A. Zgaga-Griesz, W. G. Bessler, S. K. Vashist, P. Patel, K. Achazi, J. Strotmeier, S. Worbs, M. B. Dorner, B. G. Dorner, D. Pauly, A. Rummel, G. Urban and M. Krueger, *Analyst*, 2013, DOI: 10.1039/C3AN00525A.