**Supplemental Information**

# Non-invasive single cell aptasensing in live cells and animals

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**TABLE OF CONTENTS**

## MATERIALS AND METHODS

### Reagents and small molecule drugs

All reagents were obtained from commercial suppliers and used without further purification. Oligonucleotides were purchased from IDT without further purification (Coralville, IA, USA). Aciclovir (A4669), Gardiquimod (cat# SML0877-5 mg), Resiquimod (cat# SML0196-10 mg), theophylline (cat# T1633), tetracycline (cat# 87128-25G), caffeine (cat# C0750-100G), and neomycin (cat# N1876-25G) were purchased from Millipore Sigma (Burlington, MA, USA). (6R,S)-5-formyl-5,6,7,8-tetrahydro folic acid, calcium salt (cat# 16.220-5) was purchased from Schircks Laboratories (Bauma, Switzerland). Gibco Dulbecco's Modified Eagle Medium (DMEM), Gibco antibiotic-antimycotic (100X), Gibco trypsin, Gibco fetal-bovine serum (FBS, cat# A3160702), Gibco PBS, and Lipofectamine-3000 (cat# L3000015) were purchased from Fisher Scientific. No unexpected or unusually high safety hazards were encountered.

### Genetically encoded aptamer biosensor construction

A custom-made plasmid (pDIVE), compatible for expression in zebrafish was designed and assembled for use as the backbone vector (core plasmid) for biosensor insertion. The plasmid was sequenced verified at Plasmidsaurus (Eugene, OR). Each aptamer construct was produced via overlap extension PCR, purified, and assembled into the linearized plasmid in the 3'-UTR of GFP at the XmaI restriction site using Gibson assembly (see **Figure S1, Table S1**). The Gibson mix was then transformed into competent TOP10 *E. coli* and cultured overnight at 37 °C. Colonies were picked, grown, purified, and sequence verified by Sanger sequencing using forward and reverse sequencing primers (**Table S2**).

### Cell culture and transfection

Human embryonic kidney (HEK293T) cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic (penicillin and streptomycin)-antimycotic. The cells were cultured at 37 °C in a humid atmosphere with 5% $CO_2$. $2\times10^5$ cells were seeded in each well of a 24-well plate. After 24 h, the cells were treated with drug and transfected with 0.5 µg of biosensor plasmid using Lipofectamine-3000.

### Measuring single-cell biosensor response

After 72 h of incubation, cells were detached from the culture dish by incubating with trypsin for 3 min. DMEM was then added and the cells were transferred to an Eppendorf tube. The tubes were centrifuged, then the media was removed and replaced by PBS supplemented with 1% BSA. 20,000 cells from each sample were analyzed using the BD Accuri C6 plus Flow Cytometer with the corresponding filters for GFP (ex/em: 488/510 nm) and mCherry (ex/em: 587/610 nm). Data were processed and analyzed using the standard software package FlowJo. Specifically, data were first converted into a FlowJo-compatible format (FCS3.1). Then, viable cells were isolated through two consecutive gates: 1) Scatter gate: side scatter (log) vs. forward scatter (linear); 2) Singlet gate: height vs. area of forward scatter (linear). The median EGPF level for an isolated group of viable cells under each experimental condition was exported and plotted in GraphPad Prism (v8.4.3). Each experiment was done in triplicate. The error bars represent the standard deviation for the three replicates. The limit of detection (LOD) was calculated using the following equation:[1]

$$LOD = 3.3 \times (Standard\ deviation\ of\ background)/Slope \quad (1)$$

**Live cell imaging**

$2\times10^5$ cells were seeded in each well of a 24-well plate. After 24 h, the cells were transfected with 0.5 µg of the appropriate biosensor-contained plasmid using Lipofectamine-3000. After 48 h, cells were analyzed by Evos light microscopy using the 20 X and 40X objectives. Fluorescence of GFP (ex/em: 488/510 nm) and mCherry (ex/em: 587/610 nm) were imaged, respectively.

**Bulk measurements of biosensor cellular uptake**

After 72 h of incubation, media was removed, and cells were lysed by adding Glolysis buffer (150 µL) to the 24-well plate with 5 min incubation at room temperature. All the lysed cells were transferred directly to a 96-well black plate. Bulk fluorescence for GFP (ex/em:488/510 nm) and mCherry (excitation (ex/em: 587/610 nm) was measured on a BioTek Cytation 5 Plate Reader. Ratios of GFP/mCherry were calculated and analyzed using GraphPad Prism (v8.4.3) and compared to single-cell measurements obtained using flow cytometry.

**Aptamer affinity measured using surface plasmon resonance (SPR)**

Aptamers used in the development of the biosensors were produced by in vitro transcription using T7 RNA Polymerase. DNA containing a T7 RNA polymerase promoter sequence upstream of the template, with a 24-mer poly(A) tail in the 3' end were PCR amplified using the T7 promoter primer and reverse poly T tail primer (**Table S2**). PCR products were purified using the DNA Cleanup and gel extraction kit (Monarch) following recommendations of the manufacturer. In vitro transcription from the PCR product was performed using the MEGAshortscript kit (ThermoFisher) for 16 h at 37 °C. RNA was purified using the RNA Clean & Concentrator kit (Zymo Research).

Each aptamer affinity was measured via SPR on a Biacore X100 (Cytiva) using a CM5 chip functionalized with a poly(T) as previously described.[2] In all experiments, HBS-N running buffer (Cytiva; 10 mM HEPES pH 7.4; 150 mM NaCl) was supplemented with 0.5 mM $MgCl_2$ (Life Technologies). Aptamer was captured onto the sample flow cell (flow cell 2). Increasing concentrations of the aptamer target were injected over both flow cells at a flow rate of 30 µL/min. The surface was regenerated after each cycle using 25 mM NaOH over both flow cells. Data analysis was performed using the Biacore X100 Evaluation Software, version 2.0 (Cytiva Lifesciences). Data were fit to a 1:1 binding model for kinetic analysis and steady-state affinity model for thermodynamic analysis. Reported values are the mean and standard deviation of at least three independent experiments.

**Zebrafish husbandry**

Zebrafish [WT AB (ZL1) and Casper (ZL1714)] were purchased from the Zebrafish International Resource Center (ZIRC). Adults were maintained at 28.5°C in a recirculating system (Iwaki Aquatics) on a 14:10 h light:dark cycle and fed in the morning with brine shrimp and in the afternoon with Zeigler's Adult Zebrafish Diet (Pentair Aquatic Habitats). Embryos were obtained through natural matings and cultured at 28–30 °C in E3 medium. Embryos were staged as described previously.[3] All zebrafish larvae were raised on a rotifer/brine shrimp diet starting at 5

days post fertilization (dpf) unless otherwise indicated. The National Institutes of Health Office of Laboratory Animal Welfare (OLAW) is responsible for the administration of the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Compliance with the standards of the PHS Policy is a term and condition of NIH Grants Policy Statement. East Carolina University's Animal Welfare Assurance number is A3469-01. The IACUC committee at East Carolina University, Greenville, NC, USA approved all animal procedures (AUP#W262).

**Testing the toxicity of small molecule drugs in zebrafish**

A 25 mM stock solution of theophylline was prepared in E3 medium. The fish were loaded into a 6-well plate and imaged 2 h post dosage (hpd), 4 hpd, 8 hpd, and 24 hpd. Each well in the 6-well plate had approximately 45 fish per well. Three dosages of drug (0.5 mM, 1 mM, and 2 mM) were used alongside an untreated control group. The wells that housed the fish were filled with 11 mL of E3 buffer and dosed with an amount of theophylline appropriate for the target concentration. The 6-well plate was then stored in an incubator set to 28° C. At each chosen timepoint, 10 random fish were removed and imaged. Total body length was then measured using Olympus cellSens software. Values were recorded in an Excel spreadsheet. All imaged zebrafish were used in the quantification with no exclusions. For survival analysis, 25 embryos were placed into each well of a 6-well plate and embryos were monitored for death at 2, 4, 8, and 24 hpd. Dead embryos were removed from the well. Survival curves were plotted and Kaplan-Meier analysis performed.

**Plasmid injection and transgenic zebrafish**

Plasmid DNA (pDIVE) and Tol2 mRNA were premixed and co-injected into one-cell-stage WT embryos (50 pg of plasmid; 50 pg of mRNA). [4, 5] For each plasmid, 50–100 embryos were microinjected. Fish were raised until 24 hours post fertilization and imaged for confirmation of plasmid expression using the mCherry channel. Positive embryos were raised to adulthood and mated with Casper fish to identify founders with germline transmission. The resulting F1 embryos were again outcrossed to Casper fish and a single F2 adult with monoallelic expression for each line was outcrossed to generate embryos for this study.

**Zebrafish Imaging**

Live zebrafish were mounted laterally on a glass slide using 1.5% low melting point agarose. For toxicity experiments, embryos were imaged on an Olympus SZX16 equipped with a DP80 camera. For fluorescent experiments, embryos were imaged on a Leica M165 equipped with a Flexacam C3 CMOS camera or Leica DM6 with a K8 CMOS camera. Images were saved and imported into FIJI[6] for all analysis.

**DIVE biosensor testing in zebrafish**

A 1 mM solution of theophylline was prepared in E3 medium. Fish were loaded into a 6-well plate such that each well had ten embryos. Control embryos received E3 medium only. Experimental fish received the theophylline solution. At 2, 4, 8, and 24 hpd, each individual embryo was imaged and saved as a Leica Image File (.lif) and imported into FIJI for pixel analysis. For washout experiments, 60 embryos were bathed in 1 mM theophylline for 24 hr. Ten individual embryos were randomly selected for imaging at 2, 4, 8, 12, 24, and 48 hours post washout. Pixel intensity analysis was completed.

**Fluorescence intensity quantification for Zebrafish measurements**

To quantify the pixel intensity, the embryos were dechorinated. Pixel intensities were quantified by a blinded experimenter in FIJI, using the polygon tool on the bright-field image to circumscribe the trunk of the zebrafish. The average fluorescence intensity within the same region of the corresponding fluorescent micrograph was reported. Values were recorded to an excel spreadsheet, background pixel intensity from above the trunk region were subtracted and the GFP/mCherry ratio calculated. All imaged zebrafish were used in the quantification with no exclusions.

**Statistics of zebrafish experiments**

For plasmid injections and toxicity experiments, at least two breeding tanks, each containing 2-3 males and 3-5 females from separate stocks, were set up to generate embryos. Embryos from each tank were randomly distributed across tested conditions. Unfertilized eggs and developmentally abnormal embryos were removed prior to imaging or compound treatment. For the generation of transgenic lines, at least two individual F0 fish were outcrossed to generate independent F1s. F1s were individually outcrossed to generate two independent F2 lines. Each line was tested for monoallelic expression of the transgenic and proper tissue expression throughout the entire embryo before determining the line to use for experiments. Once a line was chosen, the F2 embryos from that line were used for compound treatment. No statistical methods were used to determine sample size per condition. Values for individual fish are plotted, and each distribution was assessed using the Shapiro-Wilk test. All graphs were prepared in GraphPad Prism and statistical testing was completed in the software (Dotmatics). Individual tests are reported in the figure legends.

**Table S1.** Sequences of the DIVE plasmid components.

**Ubi Promoter**

ATTACGCCAAGCTATCAACTTTGTATAGAAAAGTTGGCTCCGAATTCGCCCTTAAACTCGAGACCAGCAAAGTTC
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TTCAGAAGATCTGGATATTCTAGCAGTTGTTCAAGCTCATGGAGGGATCAGTGACCTGATTCCACAATGACTAGG
CCTAATCCAGAAATTAGATGACTGTCAACATAAAAAGGCACAGCACTCACTAGCTGCCCTATATATTTTATTATATT
TTACATATATTATTTTATTTATTTAGCTCTGAGTGCTGTACTTTCTGGTTAAAGAAAACTGCTTACAACAGCTAACCT
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GCTGTTCACTCAGCAACAGATAGTCAGTATAAGGTCAGTGTGTCTCAAAGCAGTGCCATCTGTTTCACACATTGC
GTTCTATATATAAGTGTGCTGGTTGACACGACACTGTATAAGGCCTAGGCTAAAACACAAACAATGTAGAATGAC
ACTGTGTTTTTTTTGTAAACAAATGTTGTTTTTGGTTAAACATCTTTGTGAAAACATCCTCCTGTCATGTATTTGCT
ATATTCAAATGTTAAACCCGTGCAGAATAGAACATATACAAAAAAAAACAACACAACACATTTTTAAACATTATTA
AATATCAAGTATTGCTGGCAGTTCTGTTTCTGTTTTACAGTACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCA
GATTCCACAAGTCTGATTCACCAATAGCAAAGCGAATAAACAACCAAAGCAGCCAATCACTGCTTGTAGACTGTC
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CAAAACATGTAATTCCTAGAACATAACCATAGCAATCATTAGTTTTCAGGGTAATTATGTATTTTTAGGATTTGACT
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CGTTTAACATGGGAGAAGTGCAAAACATACATTATTGGCTAGAACATTGTAGTATTTTTTAAATGGAAATGTGTG

ATTGCTAATCTTACTTTGAATTTGTTTACAGGGATCCAAAAAGGGCGAATTCGACCCAAGTTTGTACAAAAAAGC
AGGCTGGGCTAGC

**GFP**

GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG
GGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCA
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TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGA
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CCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGAT
CACTCTCGGCATGGACGAGCTGTACAAGGGCGGTGGAAGATCTGGGAATTCAAGGCCTCTCGAGCCTCTAGAT
TCTGCAGCCCTATAGC

**mCherry**

TTACTTGTACAGCTCGTCCATGCCGCCGGTGGAGTGGCGGCCCTCGGCGCGTTCGTACTGTTCCACGATGGTGT
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GGCCTTGTAGGTGGTCTTGACCTCAGCGTCGTAGTGGCCGCCGTCCTTCAGCTTCAGCCTCTGCTTGATCTCGCC
CTTCAGGGCGCCGTCCTCGGGGTACATCCGCTCGGAGGAGGCCTCCCAGCCCATGGTCTTCTTCTGCATTACGG
GGCCGTCGGAGGGGAAGTTGGTGCCGCGCAGCTTCACCTTGTAGATGAACTCGCCGTCCTGCAGGGAGGAGT
CCTGGGTCACGGTCACCACGCCGCCGTCCTCGAAGTTCATCACGCGCTCCCACTTGAAGCCCTCGGGGAAGGA
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CAGGATGTCCCAGGCGAAGGGCAGGGGGCCACCCTTGGTCACCTTCAGCTTGGCGGTCTGGGTGCCCTCGTA
GGGGCGGCCCTCGCCCTCGCCCTCGATCTCGAACTCGTGGCCGTTCACGGAGCCCTCCATGTGCACCTTGAAGC
GCATGAACTCCTTGATGATGG

**Tol 3' arm**

AGATACGGCCACGGGTGCTCTTGATCCTGTGGCTGATTTTGGACTGTGCTGCTCGCAGCTGCTGATGAATCACAT
ACTTCCTCCATTTTCTTCCACTGATTGACTGTTATAATTTCCCTAATTTCCAGGTCAAGGTGCTGTGCATTGTGGTA
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AAAAATAATACTTAAGTACAGTAATCAAGTAAAATTACTCAAGTACTTTACACCTCTGGTTCTTGACCCCCTACCTT
CAGCAAGCCCAGCAGATCCACTAGTTCT

**Tol 5' arm**

AAGGAAGTAAAAGTAAAAGCAAGAAAGAAAACTAGAGATTCTTGTTTAAGCTTTTAATCTCAAAAAACATTAAA
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GAAGAGGTGTATTAGTCTTGATAGAGAGGCTGCAAATAGCAGGAAACGTGAGCAGAGACTCCCTGGTGTCTGA
AACACAGGCCAGAT

**Table S2.** DNA template sequences for each biosensor and primers used in this study.

| Name | Sequence |
|---|---|
| **DIVE.ON** | AAACAAACAAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGA AACAGCAAAAAGAAAAATAAAAA |
| **DIVE.OFF** | AAACAAACAAAGCTGTCACCGGATGTGCTTTCCGGTACGTGAGGTCCGTGAGGACAG AACAGCAAAAAGAAAAATAAAAA |
| **DIVE_1** | AAACAAACAAAGCTGTCACCGGAATAACCGAAGTAGTTTATGGGCTACCGAAATCCGG TCTGATGAGTCCTCGGGGTGGACGAAACAGCAAAAAGAAAAATAAAAA |
| **DIVE_2** | AAACAAACAAAGCTGTCACCGGTGCTTGGTACGTTATATTCAGCCGGTCTGATGAGTCT TGGAGAGACGAAACAGCAAAAAGAAAAATAAAAA |
| **DIVE_3** | AAACAAACAAAGCTGTCACCGGAATAGTCGATGGGTGCCACATAGCACCAGAATCCAG TCTGATGAGTCTCTCGGCAGGACGAAACAGCAAAAAGAAAAATAAAAA |
| **DIVE_4** | AAACAAACAAAGCTGTCACCGGAGCTTGTCCTTTAATGGTCCTCCGGTCTGATGAGTC CTGTAGCGGGGACGAAACAGCAAAAAGAAAAATAAAAA |
| **DIVE_5** | AAACAAACAAAGCTGTCACCGGAAAAACATACCAGATTTCGATCTGGAGAGGTGAAG AATTCGACCACCTTCCGGTCTGATGAGTCCAGGTATGAGGACGAAACAGCAAAAAGA AAAATAAAAA |
| **DIVE_6** | AAACAAACAAAGCTGTCACCGGAATACCAGCATCGTCTTGATGCCCTTGGAAGTCCGG TCTGATGAGTCCCATAAGGACGAAACAGCAAAAAGAAAAATAAAAA |
| **Forward primer** | CCTCTAGATTCTGCAGCCCTATAGCTAATCATAAATATACAAACAAACAAAGCTGTCACC |
| **Reverse primer** | TGTATCTTATCATGTCTGCTGATCAGCGGGTTTCCCGGTTTTTATTTTTCTTTTTGCTGT |
| **Forward sequencing primer** | CACAACATCGAGGACGGCAG |
| **Reverse sequencing primer** | GTTGGACATCACCTCCCACAAC |
| **T7 promoter primer** | TTCTAATACGACTCACTATAGGG |
| **Reverse poly T tail primer** | TTTTTTTTTTTTTTTTTTTTTTTTTTTT |

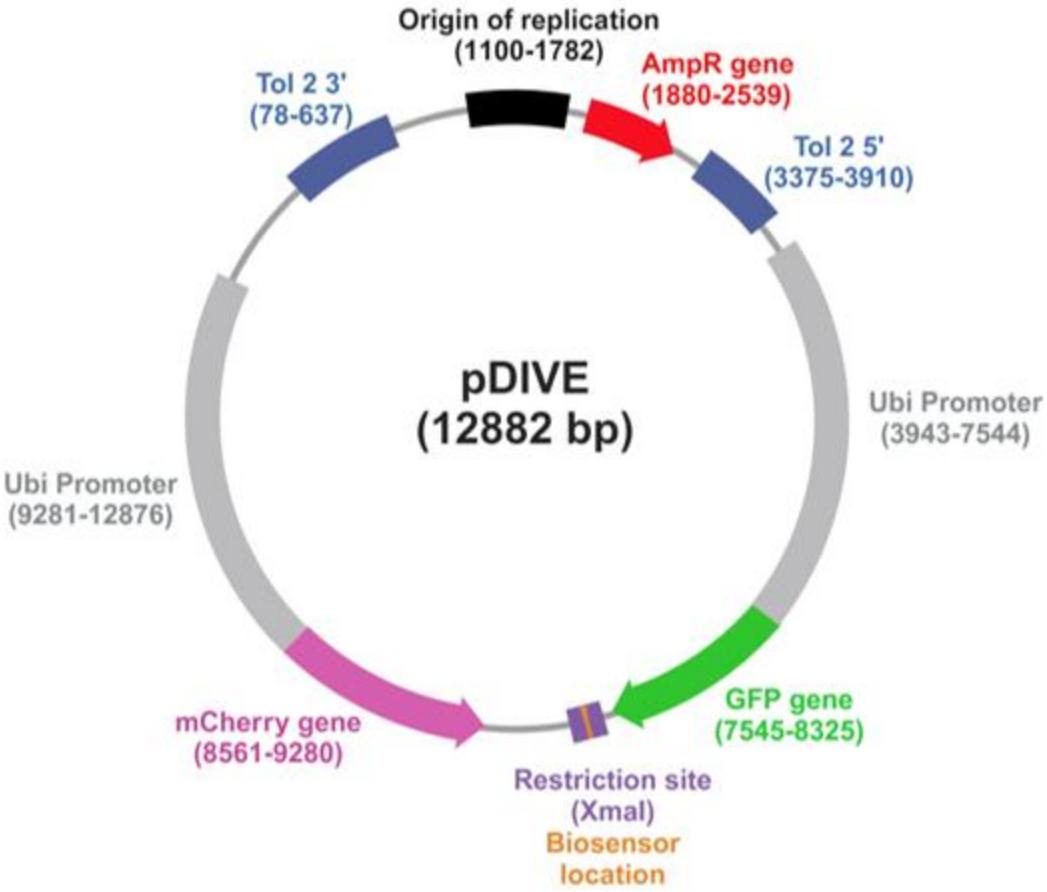


**Fig. S1. The DIVE biosensor plasmid used in this work. All sequences are compatible with zebrafish expression. Each biosensor was cloned into the XmaI restriction site.**

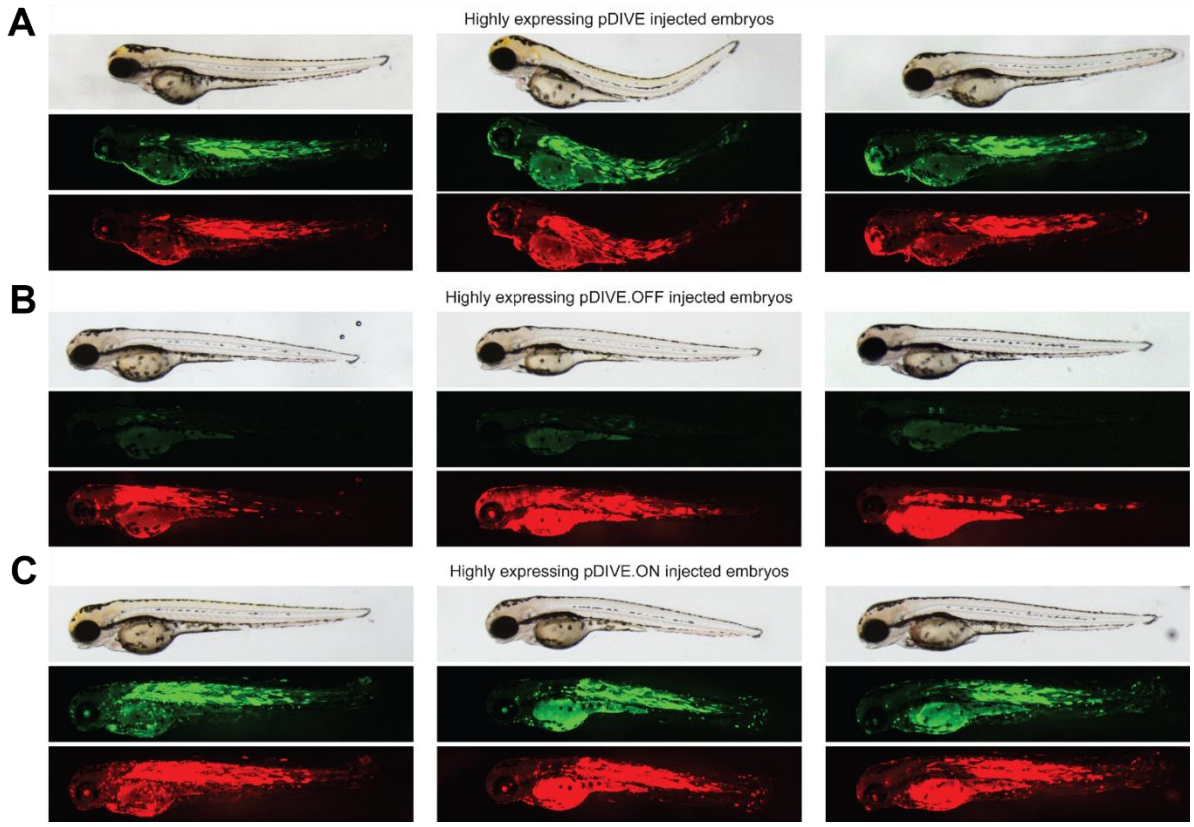


**Fig S2. Micrographs of embryos highly expressing plasmid injection.** (A) pDIVE (B) DIVE.OFF (C) DIVE.ON.

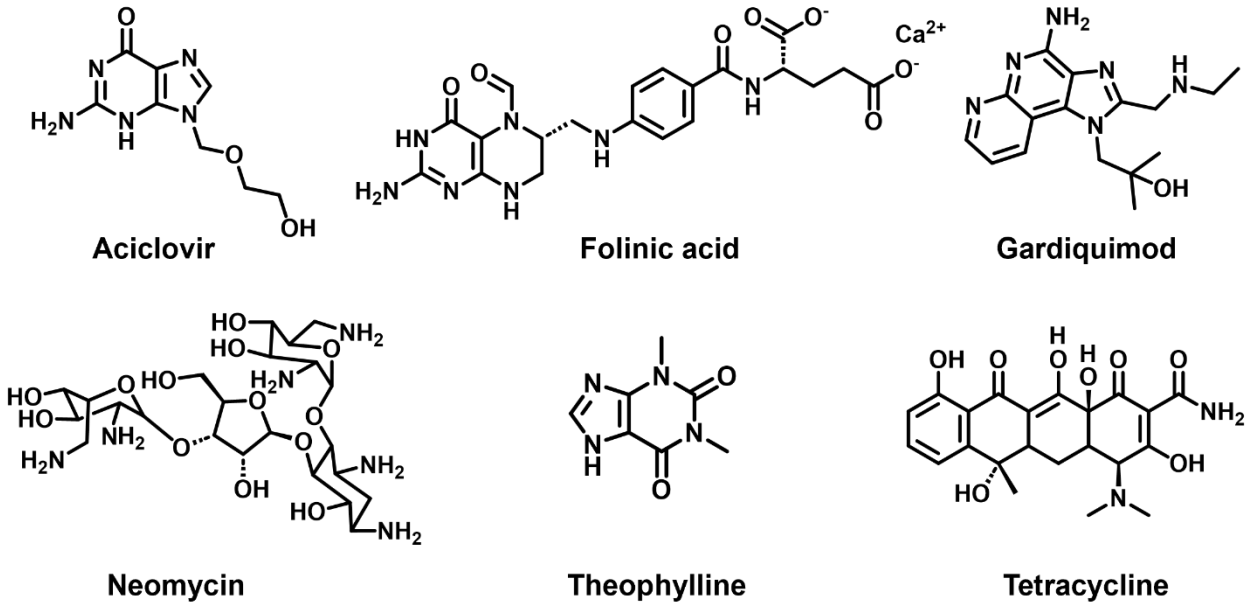


**Fig. S3. Structures for the six target drugs employed in this study.**

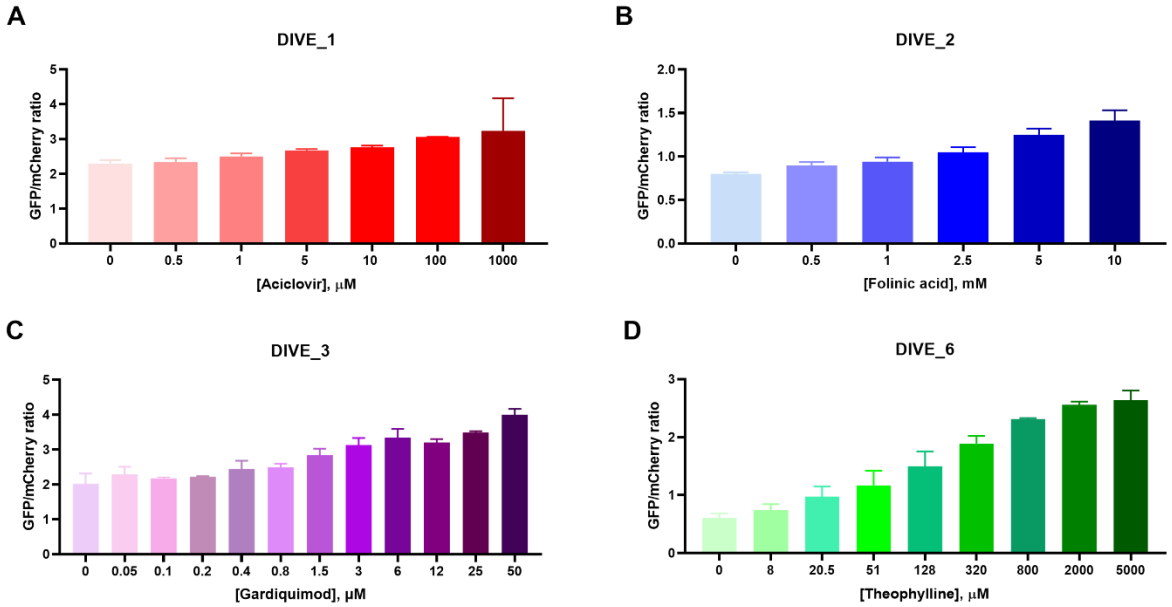


**Fig. S4**. **Comparison of biosensor activities in HEK293T cells**. Relative fluorescence of GFP/mCherry of the different aptamers biosensors with increasing concentrations of each drug: (A) DIVE_1 (B) DIVE_2 (C) DIVE_3 (D) DIVE_6. Measurements represent the mean and standard deviation of three independent biological experiments, each with technical triplicates.

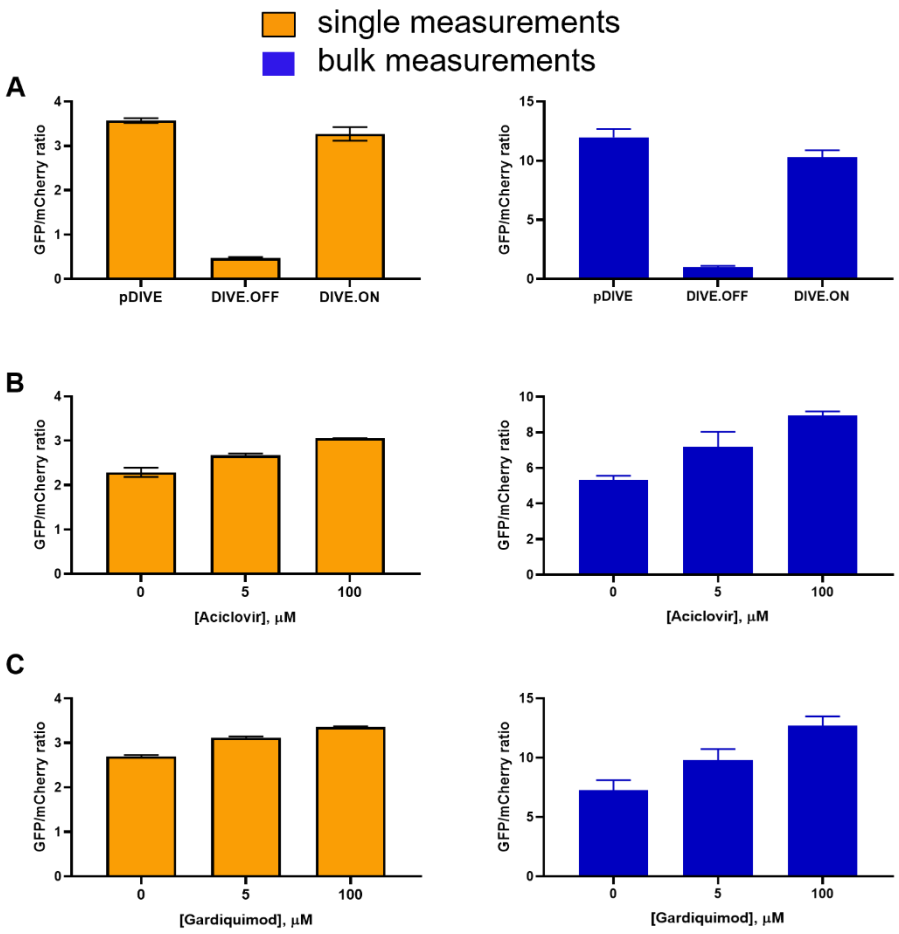


**Fig. S5**. **Comparison of single cell and bulk biosensor and control activity.** (A) Controls (pDIVE, DIVE.OFF, and DIVE.ON) (B) DIVE_1. (C) DIVE_3. The single cell measurements were obtained by flow cytometry, the bulk measurements were obtained using a plate reader. Measurements represent the mean and standard deviation of three independent biological experiments, each with technical triplicates.

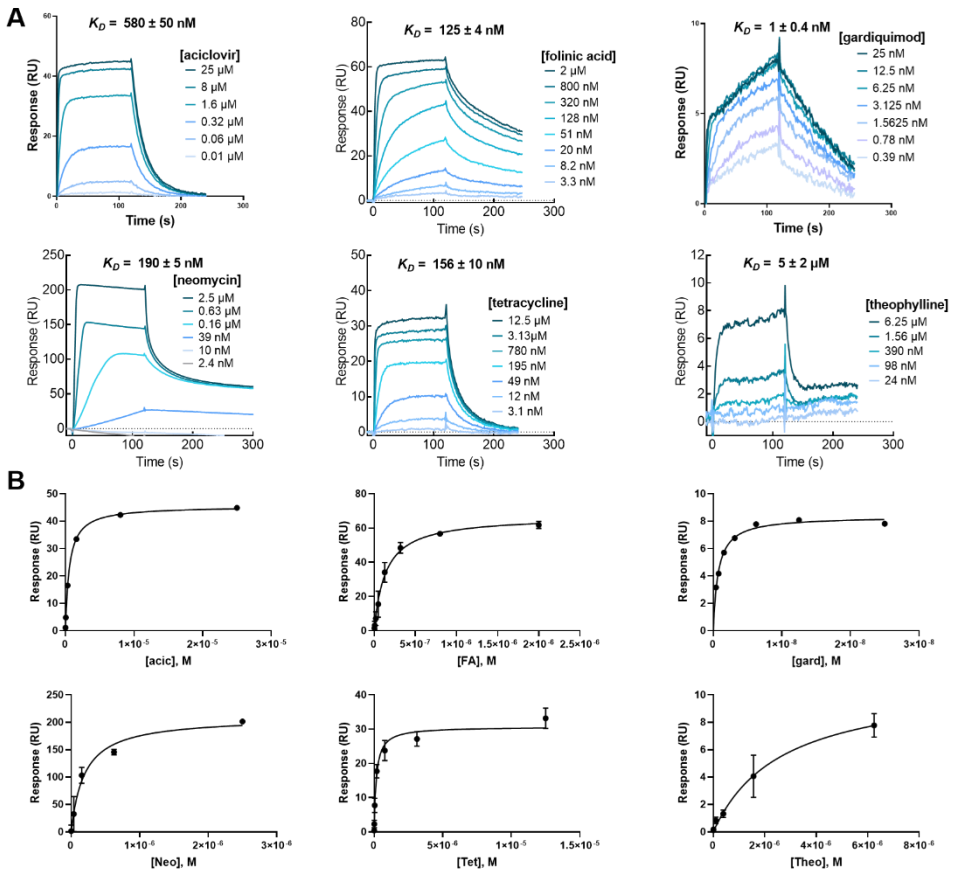


**Fig. S6. Binding of the aptamers used in the biosensors at 0.5 mM $Mg^{2+}$via surface plasmon resonance.** (A) Representative SPR sensorgrams for the six biosensors with the calculated dissociation constants. (B) Affinity curves generated from the sensorgrams.

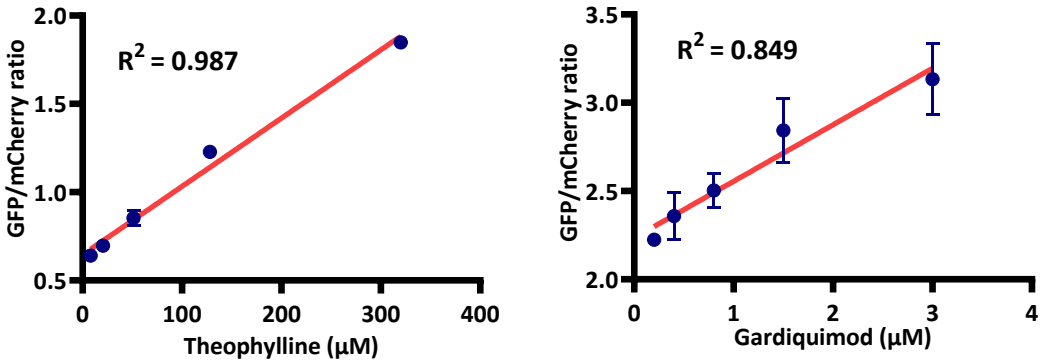


**Fig. S7. Linear drug response for DIVE_6 and DIVE_3 biosensors.**

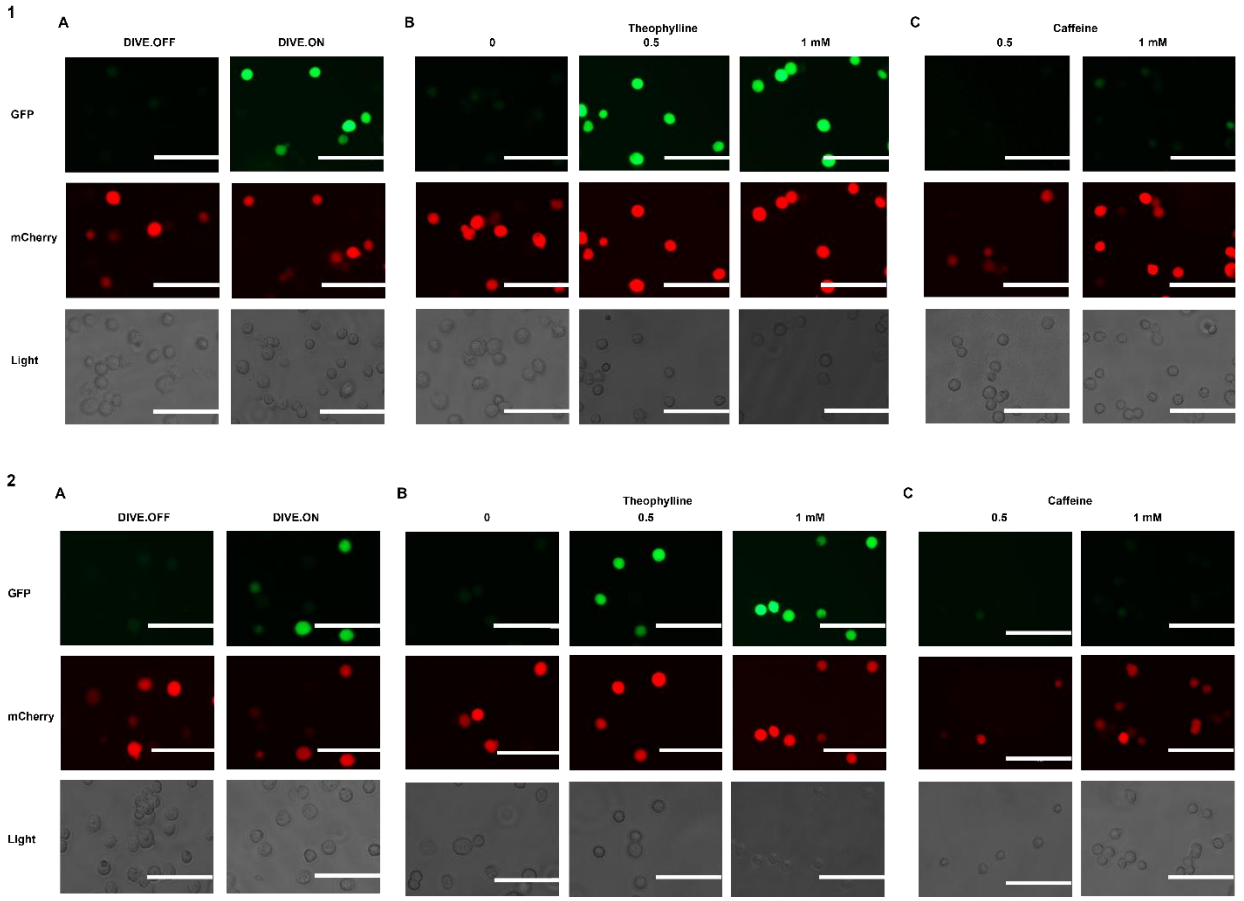


**Fig. S8 Live-cell imaging of drug uptake using DIVE aptasensors. 1 and 2** are two additional independent experiments to **Fig 4**. (A) HEK293T cells expressing DIVE.OFF and DIVE.ON controls. (B) HEK293T cells expressing DIVE_6 and treated with theophylline. (C) HEK293T cells expressing the DIVE_6 biosensor treated with caffeine. Scale bar: 100 µm.

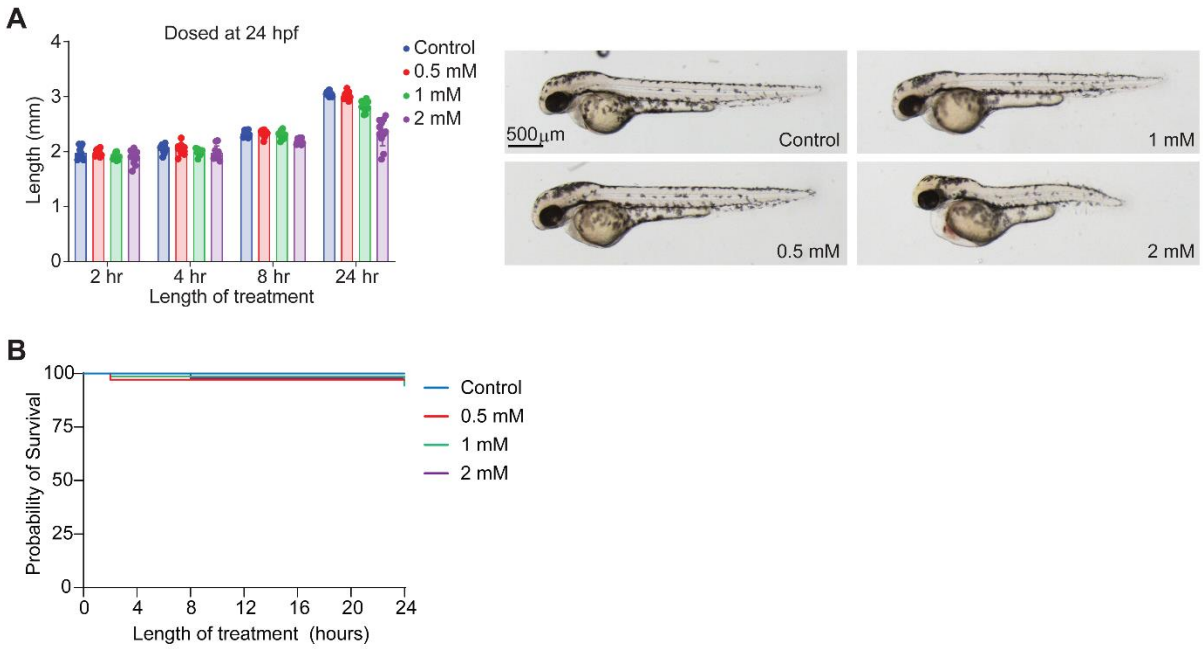


**Fig. S9. Theophylline results in low toxicity to zebrafish embryos.** WT embryos were exposed to various concentrations of theophylline at 24 hours post fertilization (hpf) and imaged over time. A) Quantification of body length and representative micrographs are shown. B) Quantification of the probability of survival over 24 hours. Orientation for all micrographs: lateral view, anterior left. Scale bar: 250 mm.

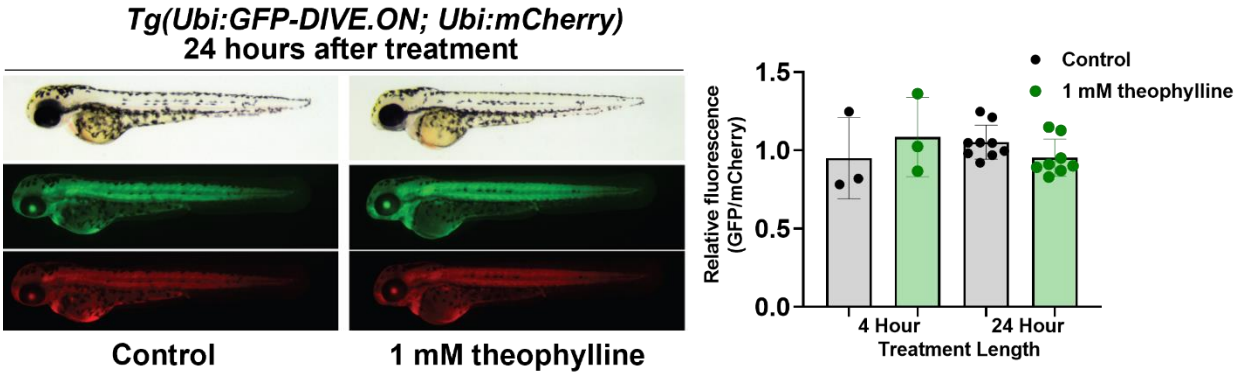


**Fig. S10. Control biosensors in zebrafish do not respond to the drug.** Representative bright field and fluorescent micrographs from 48 hpf *Tg(Ubi:GFP-DIVE.ON; Ubi:mCherry)* zebrafish embryos. Larvae were bathed in 1 mM theophylline at 24 hpf and imaged 24 h after exposure. Quantification of pixel intensity of GFP and mCherry was calculated for the trunk of the zebrafish and the GFP/mCherry ratio plotted. The average GFP/mCherry ratio ± s.d. with values for individual fish shown. p values from a two-tailed, unpaired t-test are indicated. Orientation for all micrographs: lateral view, anterior left.

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