RIBOsensor for FRET-Based, Real-Time Ribose Measurements in Live Cells

Mina Ahmadi,[a] Zhuangyu Zhao,[a] and Ivan J. Dmochowski*, [a]

[a]Department of Chemistry, University of Pennsylvania, Philadelphia, PA, USA

*Corresponding Author Email: ivandmo@sas.upenn.edu

Supporting Information

Table of Contents

Figure S1. SDS-PAGE and MALDI-MS analysis of the purified RIBOsensor. (**a**) SDS-PAGE on SEC fraction. (**b**) Native gel on SEC fraction. The fluorescence image was captured using 405 nm and 546 nm lasers on a G:Box mini 6/9 imager. (**c**) MALDI-MS result. The theoretical size of the purified RIBOsensor from the bacterial expression system is 86861.32 Da.

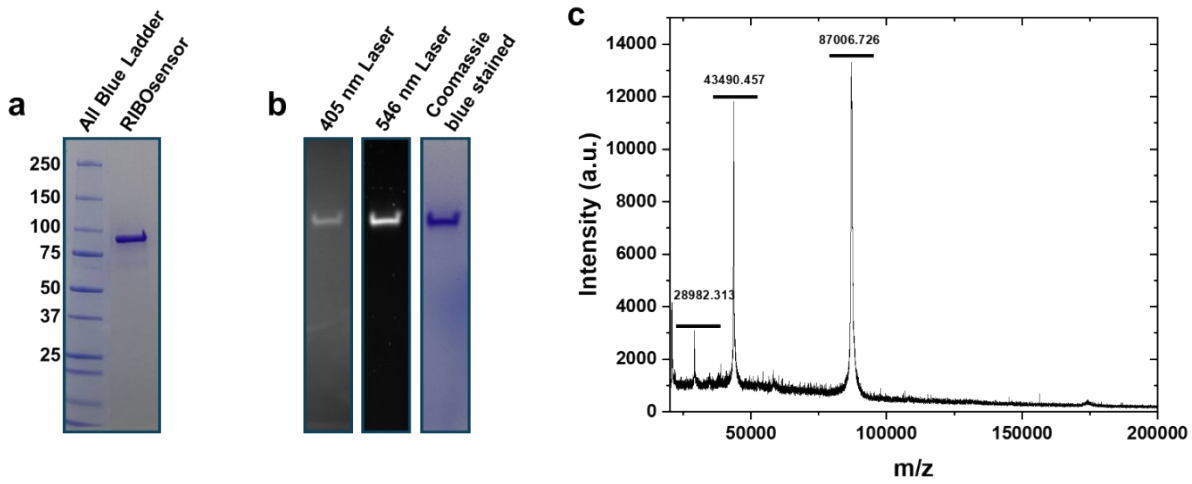

Figure S2. SDS-PAGE and MALDI-MS analysis of the purified ECFP-RBP-EYFP. (**a**) SDS-PAGE on SEC fraction. (**b**) Native gel on SEC fraction. The fluorescence image was captured using 405 nm and 546 nm lasers on a G:Box mini 6/9 imager. (**c**) MALDI-MS result. The theoretical size of the purified RIBOsensor from the bacterial expression system is 84706.81 Da.

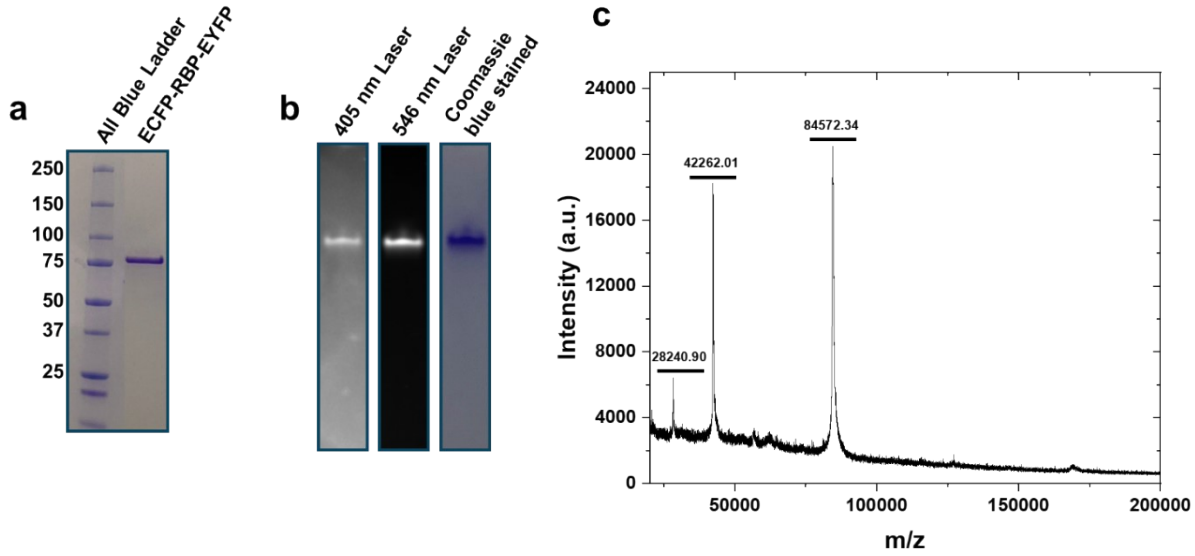

Figure S3. SDS-PAGE analysis of the purified RBP(N13.ECFP)-EYFP, and ECFP-RBP(A162.EYFP) sensors. Gels are run on the SEC fractions. The theoretical size of RBP(N13.ECFP)-EYFP and ECFP-RBP(A162.EYFP) sensors are 86849.15 and 86961.32 Da, respectively.

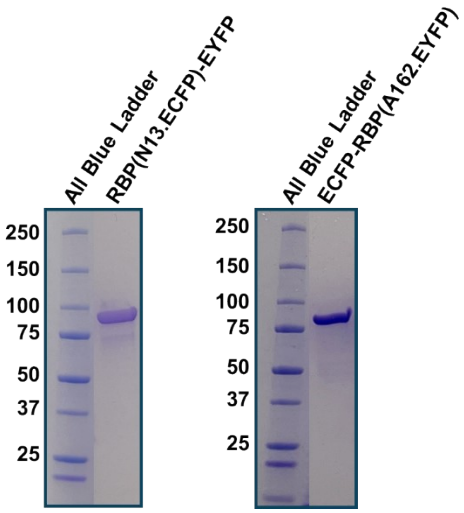

Figure S4. FRET response of ECFP-RBP(A162.EYFP) and RBP(N13.ECFP)-EYFP. 1 μM of purified ECFP-RBP(A162.EYFP) (**a**) and RBP(N13.ECFP)-EYFP (**b**) was used in the absence and presence of a saturating concentration of ribose. Samples were excited at 433 nm at room temperature using a spectrofluorometer.

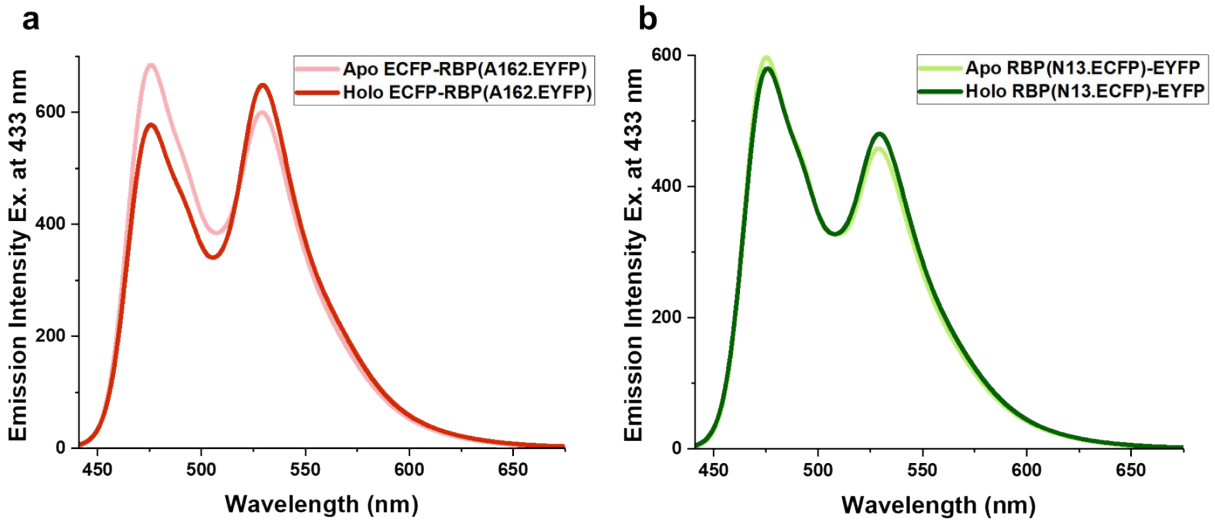

Figure S5. *In vitro* determination of the Kd for RIBOsensor and ECFP-RBP-EYFP. A sigmoidal curve (Hill equation, n = 1) was fitted to the titration data for (**a**) RIBOsensor (means of 6 independent replicates with vertical bars indicating standard deviation) and (**b**) ECFP-RBP-EYFP (means of 4 independent replicates with vertical bars indicating standard deviation) to determine the apparent dissociation constant. Titrations were performed with 0.7 µM of purified sensor and D-ribose concentrations ranging from 0.01 µM to 10 mM in PBS at room temperature. Using an excitation wavelength of 433 nm, the emission ratio of EYFP at 529 nm to ECFP at 476 nm was normalized to the no-ribose sample and plotted against ribose concentration on a logarithmic scale.

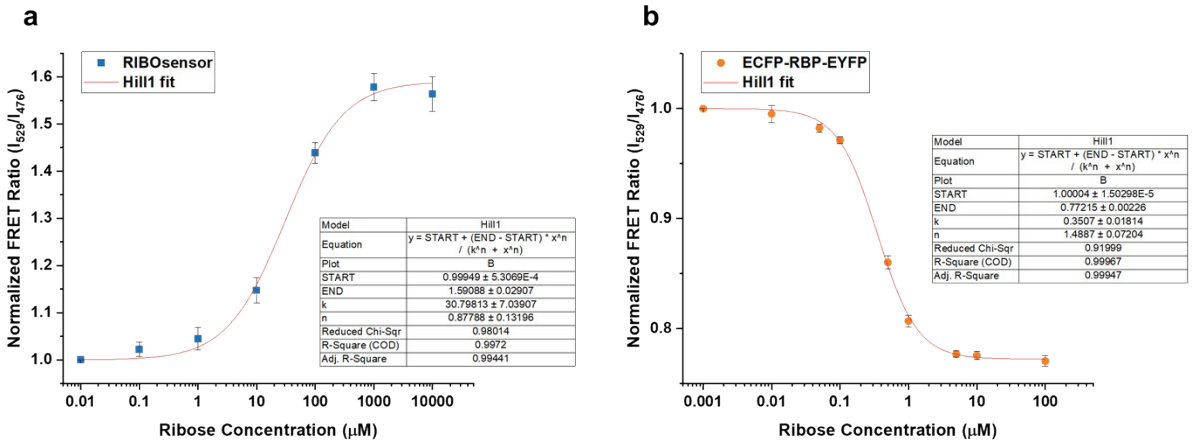

Table S1. *In vitro* determination of the Kd for RIBOsensor using Pomorski et al. fitting. The average intensities (I) at 476 nm and 529 nm, wavelength 1 and 2 respectively, and ratios are calculated from 6 replicates.

	avg	avg	avg	stdev.p	avg	stdev.p
Ligand Conc. (μM)	I(476)	I(529)	R1/2	R1/2	R2/1	R2/1
0.01	452.7112*	373.8863¥	1.212943	0.053914	0.82615	0.03847
0.1	433.4578	366.0165	1.186862	0.054262	0.844394	0.040169
1	426.6717	368.1862	1.161334	0.055772	0.863126	0.042655
10	412.7415	391.036	1.057762	0.05313	0.947861	0.04919
100	373.721	443.9722	0.84369	0.045568	1.188939	0.067988
1000	356.7308	464.7353	0.769153	0.04403	1.304686	0.079633
10000	371.2078**	478.8372¥¥	0.776709	0.049136	1.292858	0.085176

*I1u: 453
**I1b: 371
¥I2u: 374
¥¥I2b: 479

Table S2. *In vitro* determination of the Kd for ECFP-RBP-EYFP using Pomorski et al. fitting. The average intensities (I) at 476 nm and 529 nm, wavelength 1 and 2 respectively, and ratios are calculated from 4 replicates.

	avg	avg	avg	stdev.p	avg	stdev.p
Ligand Conc. (μM)	I(476)	I(529)	R1/2	R1/2	R2/1	R2/1
0.001	251.4773*	253.7438¥	0.991088	0.005136	1.009019	0.005205092
0.01	252.7085	253.6983	0.996006	0.006753	1.004057	0.006849805
0.05	252.0155	249.7818	1.008909	0.003096	0.991179	0.003048011
0.1	247.0985	242.1593	1.020466	0.007265	0.979994	0.006979019
0.5	262.8248	228.0538	1.152319	0.006325	0.867841	0.004788279
1	268.221	218.3358	1.22841	0.010817	0.814124	0.007211045
5	274.9925	215.5063	1.275985	0.002506	0.783711	0.001539253
10	269.4778	210.871	1.277899	0.003924	0.782542	0.002403561
100	277.0003**	215.282¥¥	1.286672	0.004818	0.77721	0.002912376

*I1u: 251
**I1b: 277
¥I2u: 254
¥¥I2b: 215

Figure S6. *In vitro* determination of the Kd for RIBOsensor and ECFP-RBP-EYFP using Pomorski et al. fitting. Pomorski et al. equations, provided in method section, was fitted to the titration data for (**a**) RIBOsensor (average of 6 replicates, error bars representing standard deviation) and (**b**) ECFP-RBP-EYFP (average of 4 replicates, error bars representing standard deviation) to determine the apparent dissociation constant. Titrations were performed with 0.7 µM of purified sensor and D-ribose concentrations ranging from 0.01 µM to 10 mM in PBS at room temperature. Using an excitation wavelength of 433 nm, the emission ratio of EYFP at 529 nm to ECFP at 476 nm (R2/1) or the emission ratio of ECFP at 476 nm to EYFP at 529 nm (R1/2) were calculated and plotted against ribose concentration. I_b and I_u are determined from the emission intensities of the sensor at fully bound and unbound sample (Table S1 and S2).

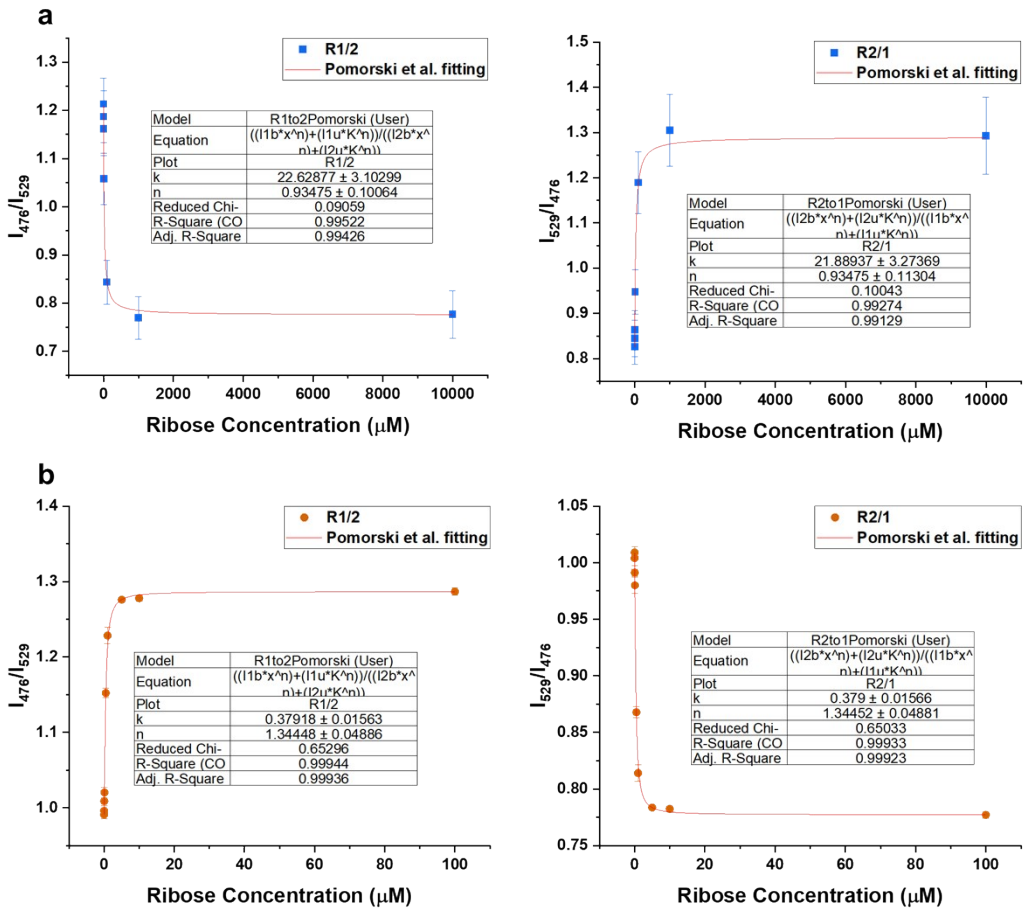

Figure S7. RIBOsensor localization in HEK293T cells. Cell images were acquired by confocal microscopy using a 60× objective 48 h post-transfection. Cells were excited with either a 458 nm laser (ECFP Em. 470-510 nm, EYFP Em. 527-567 nm) or a 515 nm laser for direct excitation of EYFP. Scale bar is 10 µm.

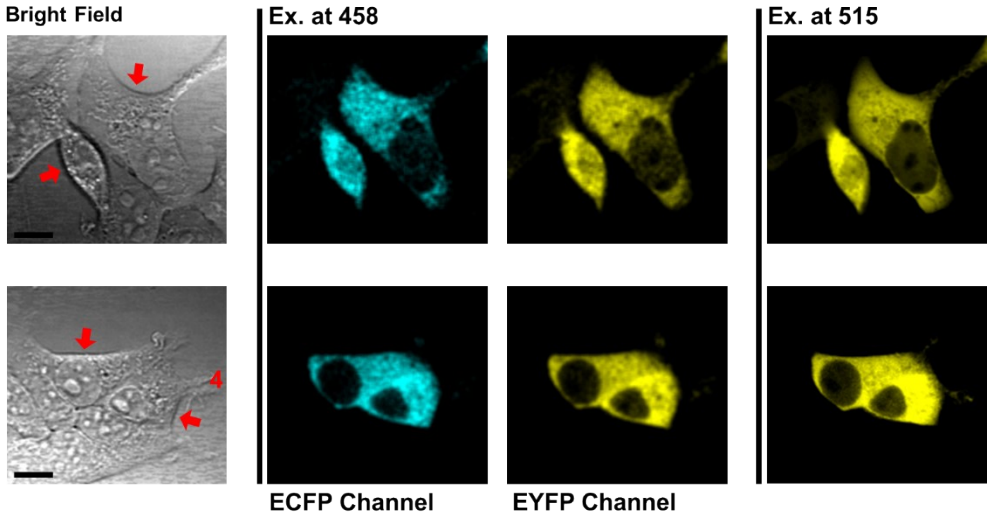

Figure S8. RIBOsensor response to various components of mammalian cell culture media. 0.7 µM of purified RIBOsensor was incubated with different components of HEK293T growth media at room temperature. The FRET ratio for each sample is normalized to the ref. value. Each additive is diluted in PBS. The data represent at least 3 independent replicates for each condition.

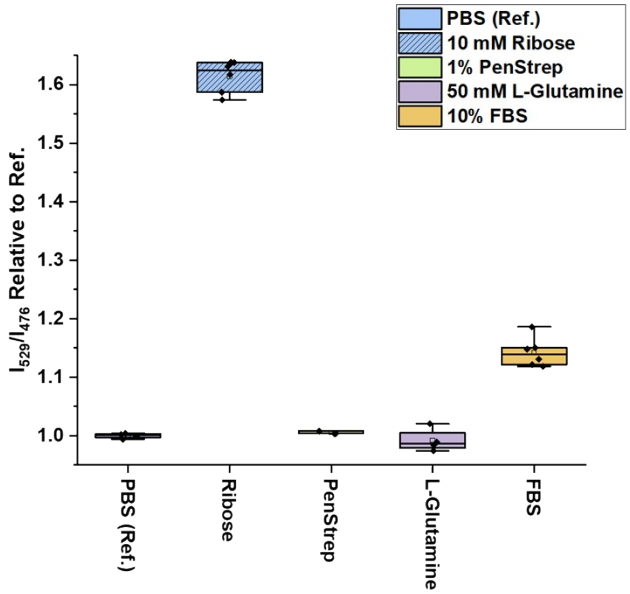

Figure S9. Processing flowchart of confocal images acquired with a 10× objective using MATLAB scripts. ECFP and EYFP channels were converted to TIFF files using ImageJ. Background signal was determined by measuring the mean emission of multiple cell-free spots in each channel using the "Measure" tool in ImageJ; this average value was used as the threshold in the MATLAB script. Subsequently, a pixel ratio image was generated by dividing the pixel values of EYFP by ECFP, referred to as the pixel ratio. For each field of view, the average pixel ratio was calculated to represent the FRET response corresponding to the cell population. Scale bar is 200 μM.

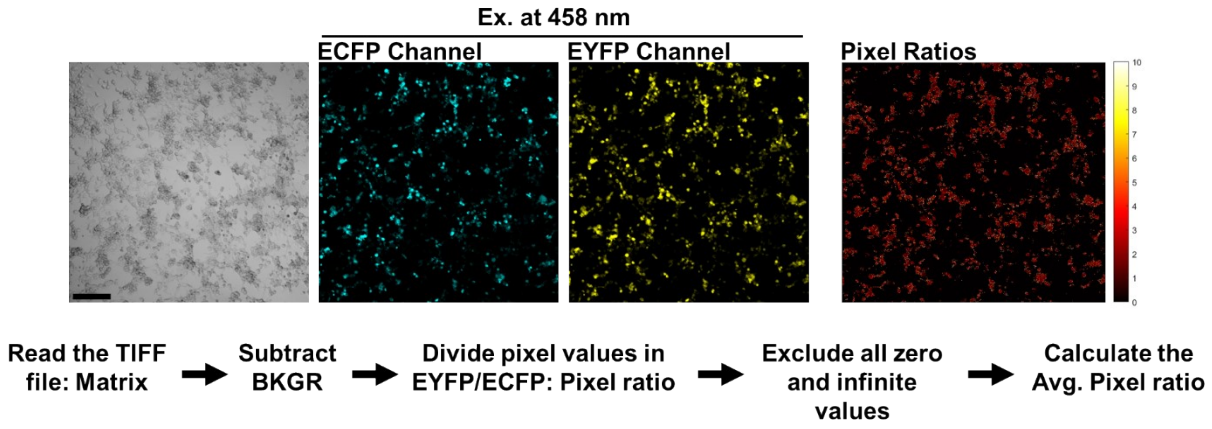

Figure S10. FRET response of HEK293T cells transfected with the RIBOsensor under starvation conditions. (**a**) Starvation protocol to identify minimal FBS and glucose content. (**b**) Outcome of reducing FBS content while maintaining glucose concentration at 25 mM. (**c**) Outcome of reducing D-glucose content with 2% FBS. The concentrations of other supplements, penicillin-streptomycin and L-glutamine, were 10 µg/L and 6 mM, respectively, for all conditions. Each point on the graph represents the average pixel ratio (see Figure S6) for a spot on the plate, collected with a 10× objective using confocal microscopy. For each media condition, at least 20 spots (i.e., 20 FRET data points) were recorded and are shown as a box plot. ****P < 0.0001.

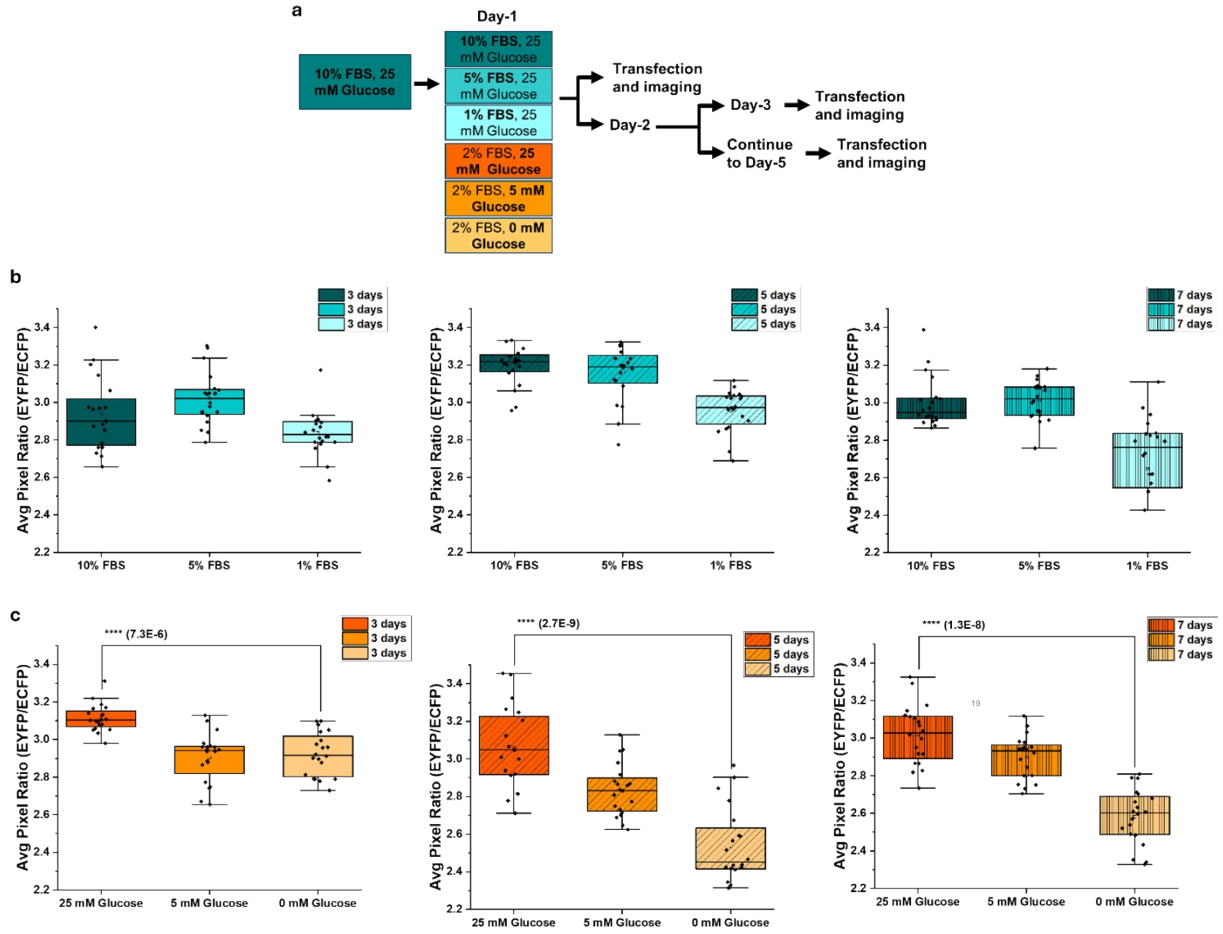

Figure S11. All images of single cell temporal response of HEK293T to the addition of D-ribose extracellularly. Spatial-temporal imaging of RIBOsensor response in live HEK293T cells upon addition of 1 mM D-ribose extracellularly in the absence (**a**) or presence (**b**) of 20 µM digitonin to permeabilize the cells. Pseudocolored ratiometric images were generated from confocal images of cells excited by 458 nm laser and simultaneously recording ECFP (470-510 nm) and EYFP (527-567 nm) channels. Cells were grown in 2% FBS and 25 mM glucose. Scale bar is 10 µm

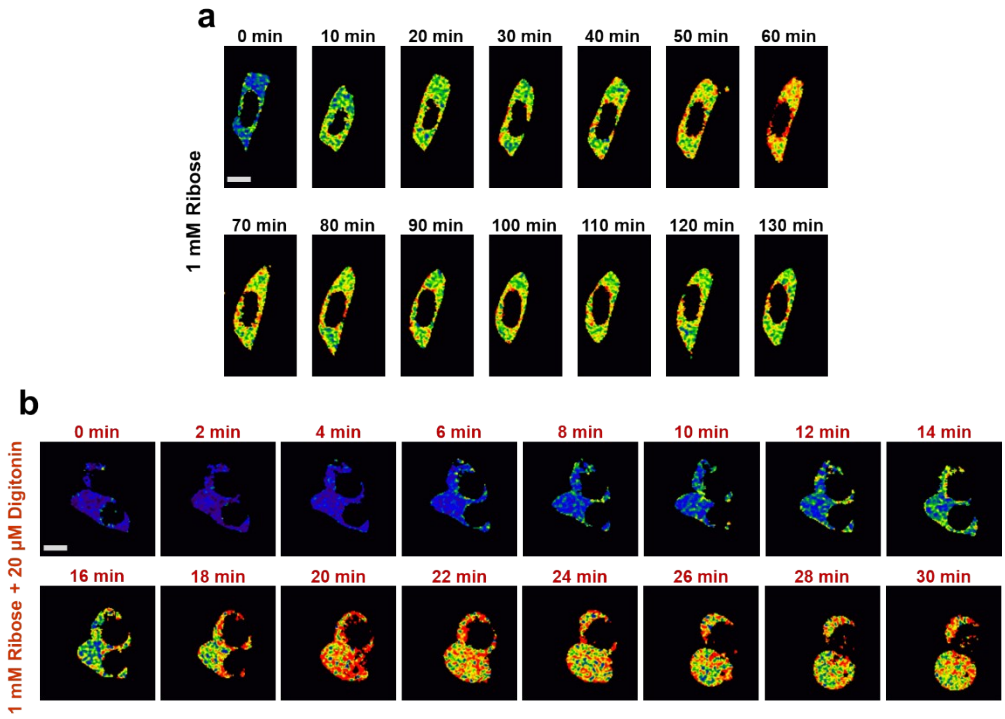

Figure S12. RIBOsensor binding kinetics *in vitro* and in HEK293T cell lysate. (**a**) The response of 0.7 µM purified RIBOsensor in PBS was measured after incubation with 1 mM D-ribose. Measurements were taken either immediately after mixing (labeled as "Just-Mixed") or after more than 15 min of incubation (labeled as "Long-Incubated"). (**b**) HEK293T cells expressing RIBOsensor were lysed in PBS, and the soluble fraction was incubated with 50 mM D-ribose. Measurements were taken either immediately after mixing (labeled as "Just-Mixed") or after more than 15 min of incubation (labeled as "Long-Incubated"). The data represent at least 3 independent replicates for each condition.

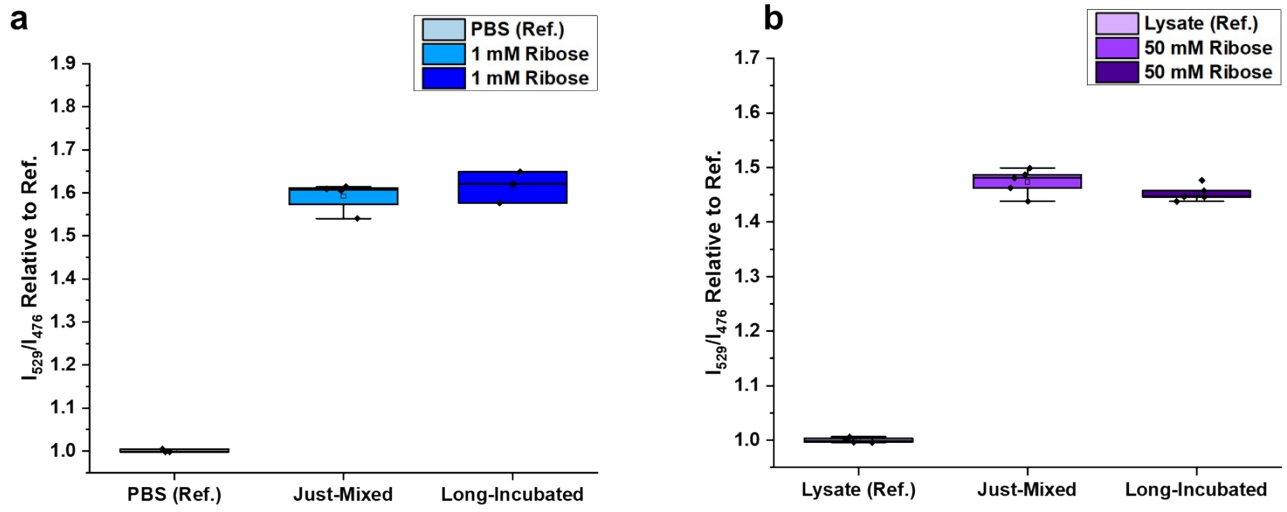

Figure S13. Photobleaching effects on ECFP, EYFP, and FRET ratio during single-cell measurements by confocal microscopy. (**a**) Quantified ECFP and (**b**) EYFP responses from two individual cells, each exposed to the light source every 10 minutes. (**c**) Maximum readout of the ECFP and (**d**) EYFP channels. (**e**) FRET response of the same cells over time.

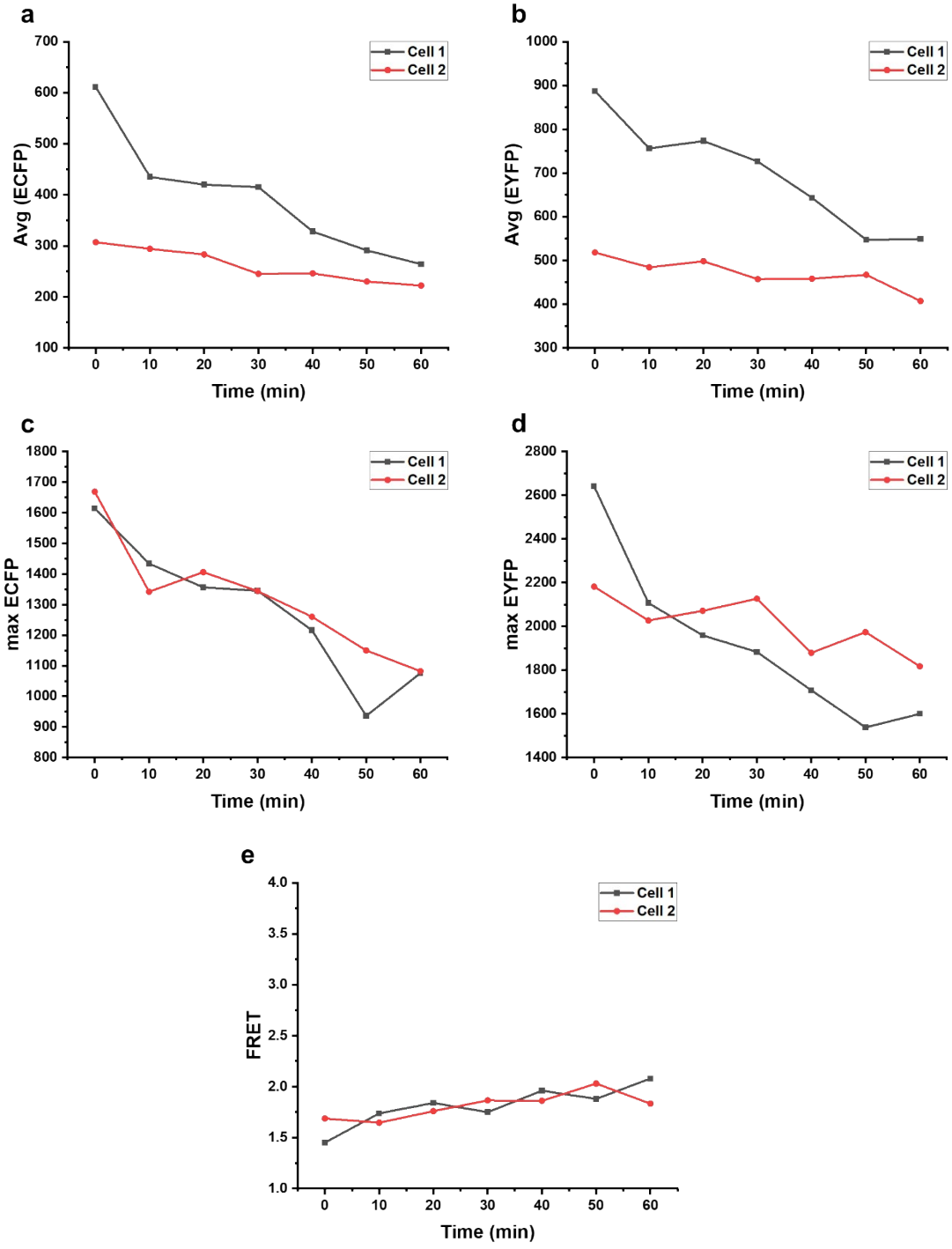

Figure S14. Temporal measurements of ribose in HEK293T cells using flow cytometry. Two more replicates of the flow cytometry data on HEK293T cells expressing RIBOsensor and grown in 2% FBS and 25 mM glucose. FRET response (Ex: 405 nm; Em: 450/50 nm for ECFP, 525/50 nm for EYFP) was measured post addition of 1 mM D-ribose up to 16 h. Saturated signal, shown in gray, is measured from cells treated with 15 µM digitonin and 50 mM D-ribose.

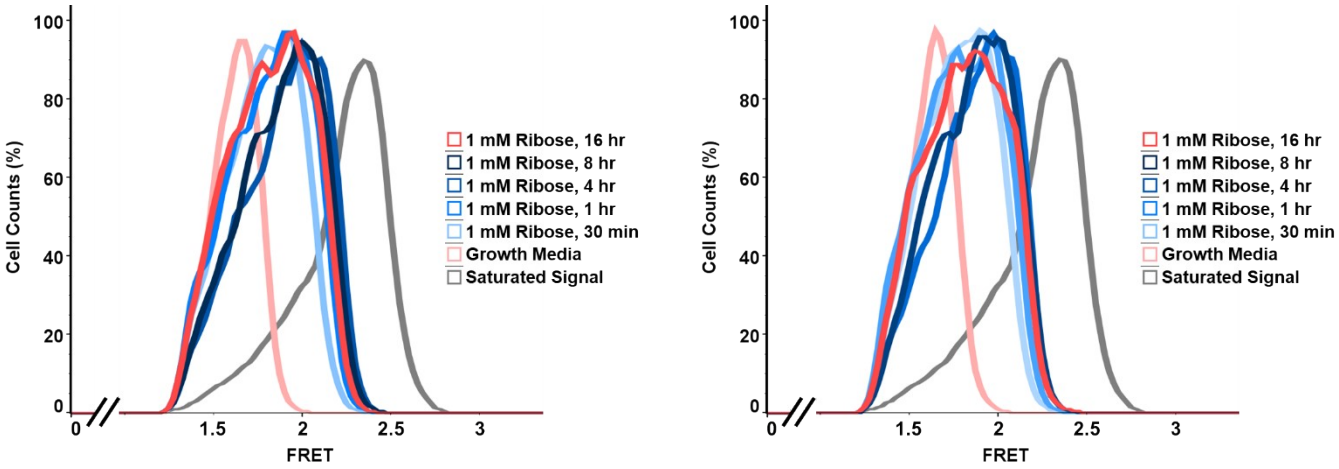

Figure S15. RIBOsensor stability in HEK293T cells post-induction. Native gels on HEK293T cells grown in 2% FBS and 5 mM glucose (labeled in orange) and HEK293T cells grown in 2% FBS and 25 mM glucose (labeled in blue). Cells were collected 24, 48, and 56 h post-induction and lysed in PBS. The fluorescence image was captured using 405 nm and 546 nm lasers on a G:Box mini 6/9 imager. Purified RIBOsensor is shown in the first lane as a control.

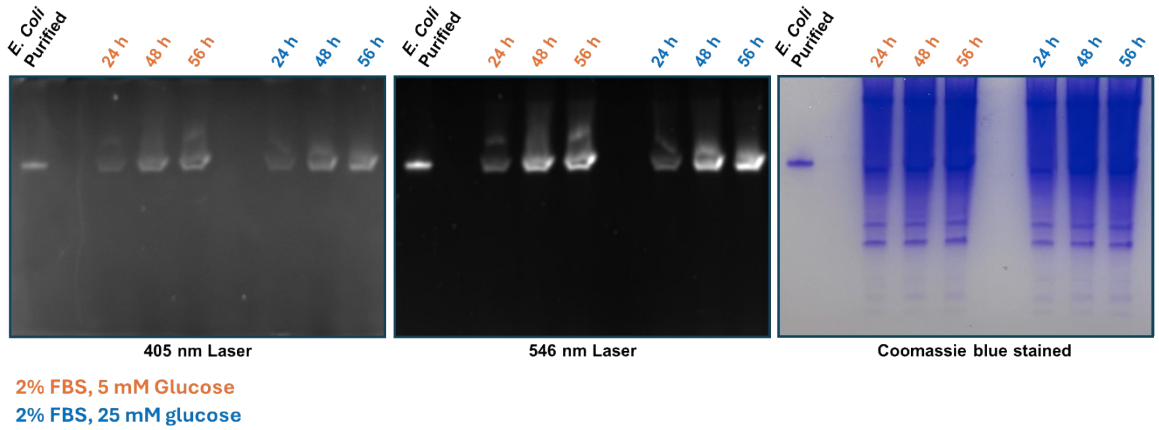

Figure S16. Comparison of different strategies to achieve minimum FRET readout in HEK293T cells. Flow cytometry data from HEK293T cells grown under various conditions: either in non-starved media (10% FBS and 25 mM D-glucose) and transfected with the ECFP-RBP(A133.EYFP)-F16A sensor, or in starved media (2% FBS and no D-glucose) and transfected with the RIBOsensor alone, or co-transfected with both the RIBOsensor and wild-type RBP.

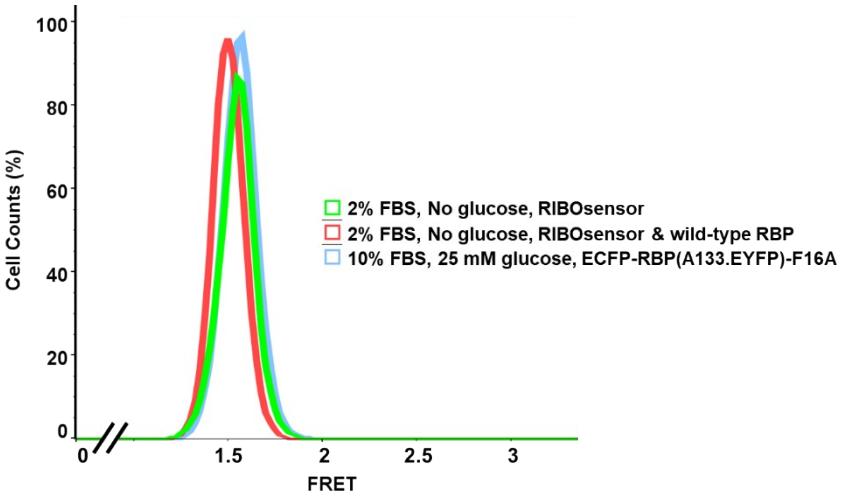

Figure S17. Generation of a non-responsive sensor. (**a**) Phe 16 is highlighted in blue in the RBP close conformation (PDB ID 2DRI). (**b**) Emission spectrum of purified 1 μM ECFP-RBP(A133.EYFP)-F16A in the absence and presence of saturating D-ribose concentration (50 mM), collected using a spectrofluorometer. Samples were prepared in PBS at room temperature. (**c**) Flow cytometry data of HEK293T cells expressing ECFP-RBP(A133.EYFP)-F16A, measured in the absence and presence of D-ribose. Cells were grown in non-starved media (10% FBS, 25 mM glucose); ribose-treated cells were incubated with 50 mM D-ribose for 1 h before analysis on the flow cytometer.

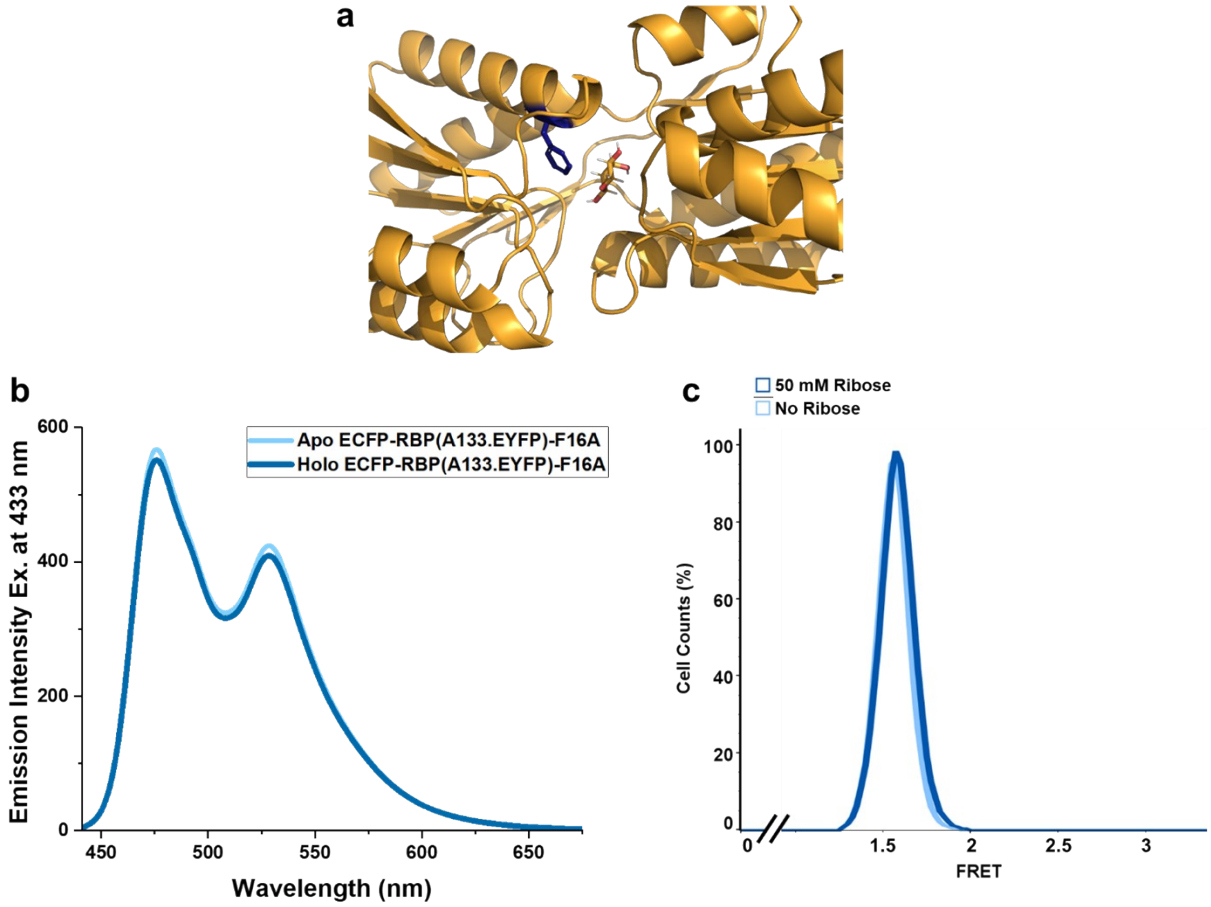

Figure S18. In situ determination of the Kd for RIBOsensor. (**a**) Response of 0.7 µM of purified RIBOsensor to 15 µM digitonin to ensure that the RIBOsensor readout is not affected by digitonin. The data represent 3 independent replicates for each condition. (**b**) Flow cytometry data representing *in situ* titration using HEK293T cells grown in starved media and expressing RIBOsensor, incubated with increasing concentrations of D-ribose in the presence of 15 µM digitonin for 30 min at room temperature. Excitation: 405 nm; Emission: ECFP 450/50 nm and EYFP 525/50 nm. (**c**) ΔFRET representation: For each cell population, the range of FRET signal (max value – min value) is defined as ΔFRET and used as the y-value in the titration curve to determine the apparent dissociation constant. (**d**) A sigmoidal curve (Hill equation, $n = 1$) was fitted to the titration data (means of 4 independent replicates with vertical bars indicating standard deviation) plotted on a logarithmic scale.