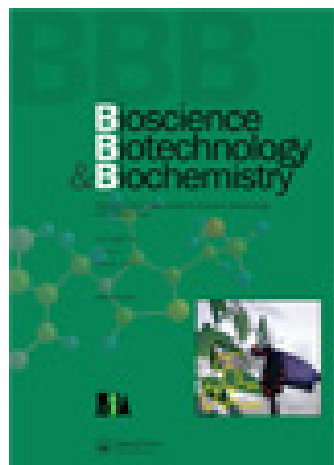




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FRET sensor-based quantification of intracellular trehalose in mammalian cells

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Note

FRET sensor-based quantification of intracellular trehalose in mammalian cells

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Trehalose acts as a stress protectant and an autophagy inducer in mammalian cells. The molecular mechanisms of action remain obscure because intracellular trehalose at micromolar level is difficult to quantitate. Here, we show a novel trehalose monitoring technology based on FRET. FLIP-suc90 μ Δ 1Venus sensor expressed in mammalian cells enables to quickly and non-destructively detect an infinitesimal amount of intracellular trehalose.

Key words: trehalose; FRET; biosensor; visual quantification; transporter

Trehalose, the non-reducing disaccharide of glucose molecules linked by an α -1,1-bond, is available as the main carbon source in micro-organisms and invertebrates.^{1,2)} It also possesses highly important properties as a chemical chaperon to prevent aggregation of proteins in conditions of stresses, such as heating, freezing, and desiccation, and it can act as an enhancer of the mTOR-independent autophagy pathway. Thanks to these properties, trehalose alleviates symptoms caused by abnormal protein aggregation, such as Huntington disease, Alzheimer disease, and prion disease in cellular and animal models.^{3–5)} The effective concentration of trehalose in cells is a range from 1 μ M to 100 mM. For example, high levels of intracellular trehalose (~20 mM) confer desiccation tolerance to Chinese hamster ovarian (CHO) and hepatoma cells^{6,7)}, whereas low levels (10–500 μ M) effectively inhibit aggregation involved in a neurological disorder, spinocerebellar ataxia type 14.⁸⁾ To study the biological function of trehalose in detail, accurate quantification of the actual cytosolic concentration of trehalose is necessary. High trehalose levels can be measured by conventional detection systems for carbohydrates, such as an HPLC equipped with sugar separation columns;^{9,10)} however, this method is unsuitable for microscale determination of trehalose because the minimum detectable quantity

is ≥ 1 mM. In addition, for accurate quantification, loss of trehalose due to enzymatic or chemical degradation in the process of extraction from the cells has to be minimized. Hence, highly sensitive and non-destructive quantification systems for trehalose in cells are desirable to overcome these difficulties.

Genetically encoded Förster Resonance Energy Transfer (FRET)-based sensors provide an attractive molecular recognition tool with high spatiotemporal resolution and sensitivity in individual cells, and are capable of quantifying and visualizing small molecules such as sugars, ions, amino acids, and other metabolites.^{11–14)} FRET sensors show spectral changes in fluorescence as a consequence of analytes to a ligand binding protein fused to two fluorophores.¹⁵⁾ The FLIP-Suc-YFP sensor (alternative acceptor, Venus¹⁶⁾) was engineered by fusion of a FRET pair of fluorescent proteins to the N- and C-terminus of the bacterial periplasmic binding protein, AtThuE, from *Agrobacterium*.¹⁷⁾ The original sucrose sensor, FLIPSuc4 μ was able to detect disaccharides such as maltose, turanose, and trehalose.¹⁷⁾ The improved sucrose sensor FLIP-Suc90 μ Δ 1Venus showed increased selectivity for sucrose. Here, we show that FLIPSuc90 μ Δ 1Venus recognizes trehalose with similar affinity as sucrose. Previous studies using this sensor had shown that sucrose did not trigger spectral changes in various human cell types expressing FLIPSuc90 μ Δ 1Venus indicating that the intrinsic sucrose uptake activities in the cells were below the detection levels.¹⁸⁾ The sensors were functional in human cells because they responded in cells co-expressing sucrose transporters of the plant SWEET family.¹⁸⁾ To detect intracellular trehalose using the FRET sensor, we co-expressed the trehalose transporter 1 (PvTRET1) which mediates trehalose influx into the cells by facilitated diffusion across the membranes down sugar concentration gradients.⁹⁾ In the present study, we characterize the properties of the sensor and perform continuous trehalose monitoring in human cell lines.

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Abbreviations: FRET, Förster Resonance Energy Transfer; FBS, fetal bovine serum; D-MEM, Dulbecco's modified Eagle medium; HBSS, Hank's buffered saline solution; HEK293T, human embryonic kidney 293T; TRET1, trehalose transporter 1.

The genetically encoded FRET-based sucrose sensor, FLIPSuc90 μ Δ 1YFP-pRSET-B was obtained from Addgene (No. 14475). The eYFP was replaced with mVenus obtained from the Miyawaki laboratory (Riken, Japan) as an alternative acceptor.¹⁶⁾ The construct of FLIPSuc90 μ Δ 1Venus was transferred into *Escherichia coli* BL21 (DE3) for bacterial protein expression. Sensor protein purifications had been described previously.¹⁵⁾ Sensors were purified using Ni-NTA chromatography (Profinity™ IMAC Ni-charged Resin, Bio-Rad, Hercules, CA). *In vitro* analyses were performed with different concentrations of trehalose in 20 mM sodium phosphate buffer, pH 7 at 25 °C. Trehalose titration curves were obtained using a fluorescence plate reader (MTP-601Lab, Hitachi High-Technologies Corp., Tokyo, Japan) with excitation at 433 nm and emission maxima of 485 and 528 nm for CFP and Venus, respectively (half band width 15 nm). The ratio of Venus/CFP fluorescence intensity was determined. The K_d of FLIPSuc90 μ Δ 1Venus for trehalose was determined by fitting ligand titration curves to a single-site binding isotherm: $S = (r - r_{apo}) / (r_{sat} - r_{apo}) = [L] / (K_d + [L])$. S means saturation, $[L]$ is the trehalose concentration, r is the ratio of observed fluorescence between Venus and CFP, r_{apo} is the ratio under trehalose-free condition, and r_{sat} is the ratio at saturation. The biological samples were prepared in three technical replicates. The K_d values were analyzed using PRISM 6 (GraphPad Software, La Jolla, CA). To test whether the sucrose sensor can report trehalose levels, we expressed FLIPSuc90 μ Δ 1Venus in *E. coli* and purified the sensor and examined the affinity for trehalose *in vitro*. FLIPSuc90 μ Δ 1Venus proteins were then transferred to microtiter plates containing various trehalose concentrations and the Venus/CFP ratio were measured (Fig. 1). The fitting ligand titration curve was characterized by a K_d of $100.3 \pm 16.9 \mu\text{M}$ for trehalose, which is similar to that for sucrose determined in previous *in vitro* analyses.¹⁷⁾ We conclude that FLIPSuc90 μ Δ 1Venus allows detection of trehalose in a range from ~10 to ~1000 μM .

The pPvTRET1-IRES2-AcGFP1 (PvTRET1 accession No. AB272983) was previously constructed in a mammalian cell expression vector.⁹⁾ The vector was

digested with *Bam* HI and *Not* I restriction enzymes to remove the region encoding AcGFP1, and filled in both of the digested termini with Klenow polymerase. pPvTRET1-IRES2 was created by self-ligation of the treated fragment. An amplified DNA fragment for FLIPSuc90 μ Δ 1Venus with primers (forward primer, 5'-ATG CGG GGT TCT CAT CAT CAT C-3' and reverse primer, 5'-TTA CTT GTA CAG CTC GTC CAT G-3') was directly integrated into the mammalian cell expression vector pcDNA3.1/V5-His (Life technologies, Carlsbad, CA).

Human embryonic kidney 293T (HEK293T) cells were cultured in D-MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma-Aldrich) at 37 °C, an atmosphere of 5% CO₂, and 95% relative humidity. HEK293T cells were seeded on the 35 mm cell culture dishes, and incubated for 24 h. FLIPSuc90 μ Δ 1Venus-pcDNA3.1 and pPvTRET1-IRES2 were co-transfected to the cells using Opti-MEM® (Life technologies) containing the transfection reagent, FuGENE® HD (Promega, Madison, WI) according to the manufacturer's protocol. The fluorescent imaging analyses were performed at 48 h after the transfection. HEK293T cells expressing FLIPSuc90 μ Δ 1Venus without PvTRET1 were analyzed for the endogenous disaccharide uptake activities.

The FRET analysis system equipped with a circulatory buffer flow system was described previously.¹⁹⁾ Cells co-expressing sensor and transporter were cultured on glass bottom dishes or glass coverslips. Before the sugar monitoring assays, cells were rinsed with Dulbecco's PBS (Sigma-Aldrich), then media were replaced with fresh Hank's buffered saline solution (HBSS; combining 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, and 0.4 mM MgSO₄, pH 7.4). The cytoplasmic fluorescent emission intensities were monitored under the excitation of a confocal microscopy (TCS SP8, Leica microsystems, Germany) installed with filters (CFP excitation 430/20, CFP emission: 475/20, Venus emission: 530/15), using a 40x objective (HC PL APO CS2, 40x/1.30 oil). The pinhole size was set to 65.3 μm . Cells were perfused with HBSS containing 5–40 mM disaccharides. Imaging data were analyzed using the software, Leica Application Suite Advanced Fluorescence (LAS AF, Leica). Fluorescence intensities of the cytoplasmic Venus/CFP ratio in HEK293T cells expressing FLIPSuc90 μ Δ 1Venus were monitored during the addition of trehalose to the chamber (Fig. 2(A)). No significant changes in the fluorescence intensity ratio were detected in the cells, indicating that intrinsic trehalose influx was not induced in HEK293T cells. Thus, endogenous trehalose uptake activities in HEK293T cells must be below the range detectable by the sensor. These findings are consistent with results from *Xenopus* oocytes and mammalian cells indicating low or no significant uptake capacity for trehalose.^{9,10)} To corroborate these findings, we co-expressed PvTRET1, which can promote trehalose influx into cells with the FLIPSuc90 μ Δ 1Venus sensor. When the fluorescence intensity ratio was monitored during the addition of trehalose to the buffer, rapid changes in the intensity ratio was detected immediately

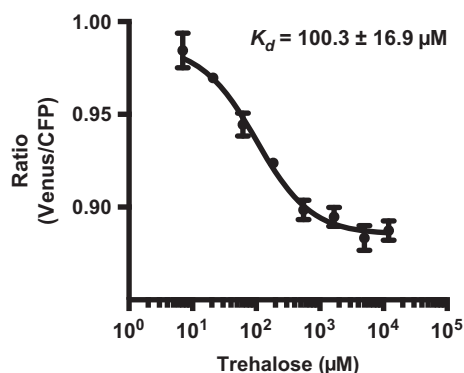


Fig. 1. *In vitro* characterization of FLIPSuc90 μ Δ 1Venus for trehalose.

Notes: The dependency of trehalose concentration is represented as a Venus/CFP ratio. The fitting titration curve to a single-site binding isotherm is described, and Values and error bars show means $\pm$ SEM ($n = 3$).

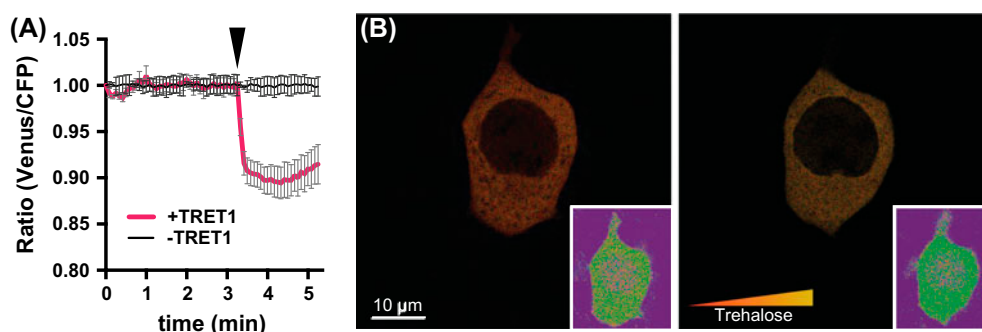


Fig. 2. Intracellular trehalose monitoring by FLIPSuc90 Δ 1Venus.

Notes: The intracellular trehalose was observed using HEK293T cells co-expressing FLIPSuc90 Δ 1Venus and PvTRET1 at 48 h after transfection. (A) The cytoplasmic fluorescent ratio is shown as Venus/CFP ratio, HEK293T cells co-expressing FLIPSuc90 Δ 1Venus and PvTRET1 (magenta), and expressing FLIPSuc90 Δ 1Venus (black), respectively. The temporal fluorescent ratio was monitored during perfusion with HBSS buffer. Arrowheads show a time-point in 40 mM trehalose. Values and error bars represent means $\pm$ SEM ($n = 8$). (B) The visualization of trehalose by the fluorescence of the FRET sensor. The fluorescence under the trehalose-free condition is shown (left), and that under the buffer containing 40 mM trehalose (right). Each inset: Cytoplasmic Venus/CFP ratio. The time-lapsed ratio movie is shown as the supplemental movie 1.

following trehalose addition (Fig. 2(A)). The FRET sensor enabled to detect and visualize intracellular trehalose in individual human cells (Fig. 2(B)), Supplemental movie 1). The maximum of fluorescent ratio changes of the sensor caused by PvTRET1-mediated uptake of trehalose were reached after 0.25 min, indicating that PvTRET1 is capable of rapid uptake. The advanced monitoring tool for trehalose would be possible to detect a small amount of ones in a few seconds and in localized spaces such as nucleus, cytoplasmic organelle, or transmembranes.

We monitored temporal trehalose levels in the individual cells expressing the FRET sensor equipped with the trehalose perfusion system, and also evaluated transport activities by PvTRET1 (Fig. 3(A)). PvTRET1 appears to function as a uniporter.⁹⁾ Moreover, human kidney cells such as HEK293T express trehalase activity.²⁰⁾ We therefore tested whether the trehalose accumulation was reversible. Removal of substrate after addition showed clear reversibility, which can be due to either metabolism or efflux (Fig. 3(A)). Intracellular trehalose concentrations are calculated as follows: $[\text{Trehalose}]_i = K_d (r - r_{\min}) / (r_{\max} - r)$. K_d value was obtained in the analysis under *in vitro* conditions as indicated above, r is the measured ratio, $r_{\min}$ is the minimum ratio with unbound form of FLIPSuc90 Δ 1Venus, and $r_{\max}$ is

the maximum ratio change of the sensor. The sensor response was concentration-dependent. Full saturation was obtained at an external supply of 40 mM, half saturation at 5–10 mM. Given that the sensor itself has a K_d for trehalose of $\sim 100 \mu\text{M}$, uptake of trehalose is by PvTRET1 is limiting, or incoming trehalose is at least in part metabolized. The intracellular trehalose concentration was estimated based on the assumption that the sensor is saturated at a supply level of 40 mM, and that the sensor affinity in the cytosol of HEK293T cells is comparable to that *in vitro* (Fig. 3B). When cell were perfused with 5, 10, and 40 mM trehalose, the calculated concentrations of intracellular trehalose were $84.4 \pm 0.3 \mu\text{M}$, $245.8 \pm 2.5 \mu\text{M}$, and $2.6 \pm 0.3 \text{ mM}$, respectively. The present study demonstrates that the sucrose sensor FLIPSuc90 Δ 1Venus recognizes trehalose with approximately equal affinity as sucrose. We also demonstrate that the sensor is suitable for monitoring cytosolic trehalose accumulation and elimination with high temporal and spatial resolution.

We propose that the FRET sensor is applicable for studying the physiological role of trehalose under various conditions such as the alleviating effect of the improper ordered protein aggregates or prevention of osteoporosis.^{8,21)}

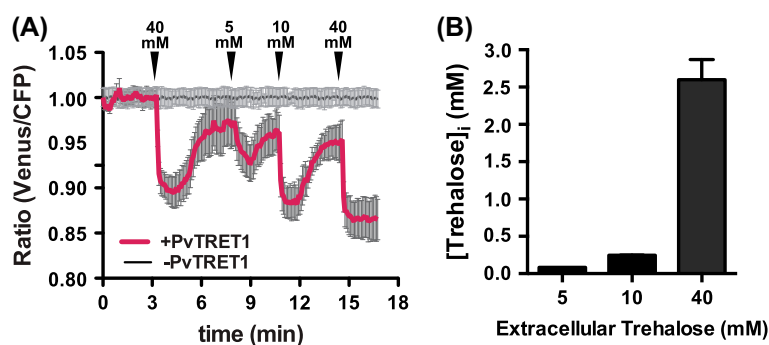


Fig. 3. Sensitivity of FLIPSuc90 Δ 1Venus in cytoplasm.

Notes: (A) The cytoplasmic fluorescent ratio is shown as Venus/CFP ratio in HEK293T cells co-expressing FLIPSuc90 Δ 1Venus and PvTRET1 (magenta), and expressing FLIPSuc90 Δ 1Venus as a control, respectively. Each arrowhead represents at a time-point perfused the HBSS buffers in various trehalose concentrations. The error bars showed $\pm$ SEM ($n = 8$). (B) Quantification of intracellular trehalose in HEK293T cells. $[\text{Trehalose}]_i$ means the concentration in a cell. Values and error bars indicate means $\pm$ SEM ($n = 3$).

Author contribution

S.K., W.B.F., and T.K. designed research; S.K., B.-H.-H., and T.K. performed research; S.K., B.-H.-H., R.S., W.B.F., and T.K. analyzed data; S.K., W.B.F., and T.K. wrote the paper.

Supplemental material

The supplemental material for this paper is available at <http://dx.doi.org/10.1080/09168451.2015.1069699>.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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