Supporting Information to Accompany "**A Two-Photon Turn-On Probe for Glucose Uptake**"

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Synthesis and Material. A[1] and AH1[2] were prepared by the literature methods. Synthesis of AS1 is described below.
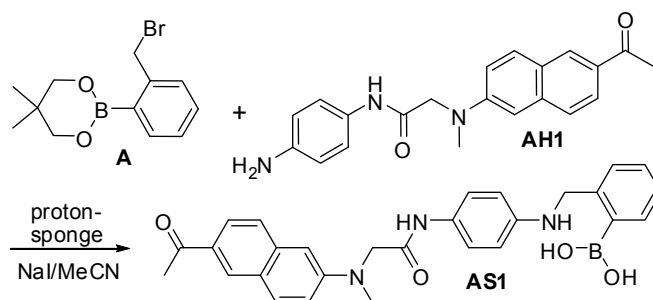
A mixture of **A** (100 mg, 0.35 mmol), AH1 (242 mg, 0.70 mmol), proton-sponge (220 mg, 1.03 mmol), and NaI (40 mg, 0.27 mmol) in dry acetonitrile (20 mL) was stirred for 30 min at room temperature under N_2 and refluxed for 8 h. The resulting mixture was cooled to room temperature, filtered and the filtrate was extracted with ethyl acetate and brine, and concentrated in vacuo. The crude product was purified by column chromatography using $CHCl_3$/MeOH (10:1) as the eluent. Yield 52 mg (31 %); mp 189 °C; ^{1}H NMR (400 MHz, DMSO d_6): δ 9.68 (s, 1H), 8.34 (s, 1H), 8.12 (s, 2H), 7.81 (d, 1H, J = 9 Hz), 7.71 (d, 1H, J = 9 Hz), 7.57 (d, 1H, J = 9Hz), 7.43 (d, 2H, J = 8 Hz), 7.12 (m, 3H), 6.97 (m, 2H), 6.85 (s, 1H), 6.41 (d, 2H, J = 8 Hz), 4.67 (s, 2H), 4.62 (br s, 1H), 4.16 (s, 2H), 3.06 (s, 3H), 2.52 (s, 3H); ^{13}C NMR (100 MHz, DMSO d_6): δ 197.2, 167.4, 149.9, 149.5, 144.9, 142.3, 137.2, 133.8, 130.7, 130.6, 130.2, 129.0, 128.1, 126.0, 125.6, 124.8, 124.7, 124.1, 121.1, 116.2, 112.0, 104.9, 59.8, 55.4, 54.7, 26.5 ppm; Anal. Calcd for $C_{28}H_{28}BN_3O_4$: C, 69.87; H, 5.86; N, 8.73. Found: C, 70.05; H, 5.99; N, 8.58.

Water solubility. Small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0×10^{-3} M). The solution was diluted to ($6.0 \times 10^{-3} \sim 6.0 \times 10^{-5}$) M and added to a cuvette containing 3.0 mL of H_2O by using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2 %.[3] The plot of fluorescence intensity against the dye concentration was linear at low concentration and showed downward curvature at higher concentration (Figure S1). The maximum concentration in the linear region was taken as the solubility. The solubility of AS1 in water is 3.0 μM.
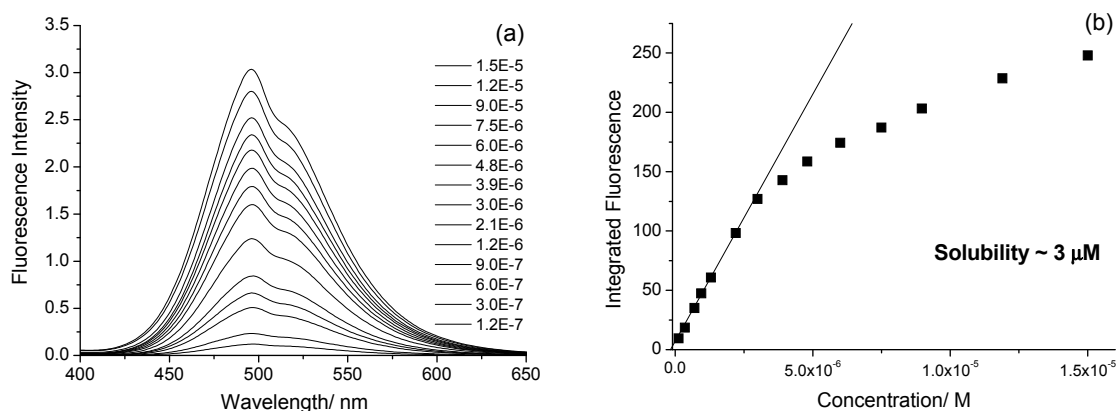

Figure S1. (a) One-photon fluorescence spectra and (b) plot of fluorescence intensity against dye concentration for AS1 in H_2O. The excitation wavelength was 365 nm.

Spectroscopic measurements. Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and fluorescence spectra were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 as the reference by the literature method. [4]
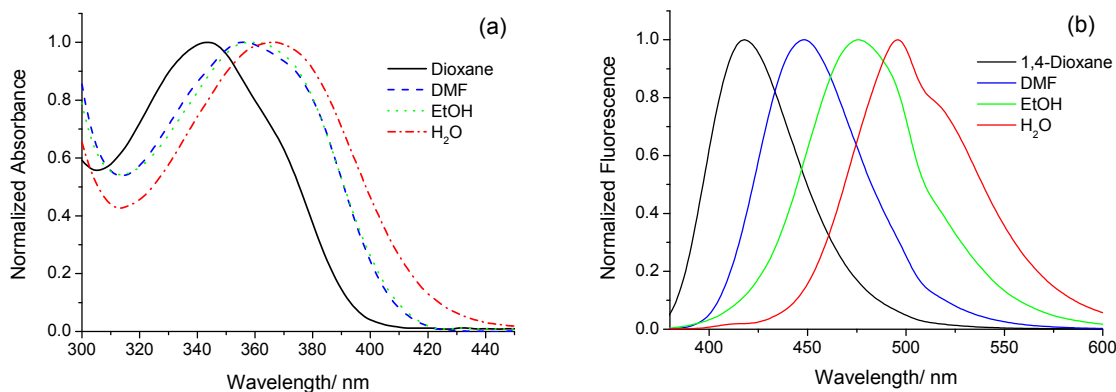

Figure S2. (a) Normalized absorption and (b) emission spectra of AS1 in 1,4-dioxane, DMF, EtOH, and H_2O.

Table S1. Photophysical properties of AS1 in various solvents.

compound	Solvent (E_T^N)[a]	$\lambda_{max}^{(1)}$, nm[b]	λ_{max}^{fl}, nm[b]	Φ[c]
AS1	1,4-dioxane (0.164)	344	418	0.34
	DMF (0.386)	356	448	0.11
	EtOH (0.654)	360	476	0.08
	H_2O (1.000)	366	496	0.04

[a] The numbers in the parenthesis are normalized empirical parameter of solvent polarity.[5] [b] λ_{max} of the one-photon absorption and emission spectra in nm. [c] Fluorescence quantum yield, ± 15 %.

pK_a value. A 3.0 µL of the stock solution of AS1 in DMSO (1.0×10^{-3} M) was added to a cuvette containing 3.0 mL of universal buffer solution[6] (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, 0.1 M KCl, pH 3.2-10.5) by using a microsyringe and the fluorescence intensity was measured as a function of the pH. The pK_a value was estimated from the increase in the integrated area of the fluorescence spectra with pH 3.2-10.5 by using the relationship, $\log[(I_{max} - I)/(I - I_{min})] = pH - pK_a$.[7] The calculated p$K_a$ value of AS1 is 5.3 ± 0.02.
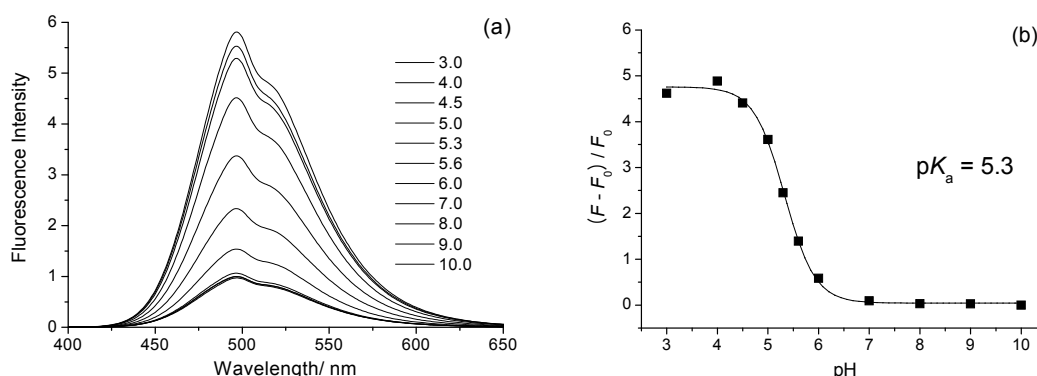

Figure S3. (a) One-photon emission and (b) titration curve of AS1 as a function of pH (3.2-10.5) in universal buffer. The excitation wavelength was 365 nm.

Determination of Apparent Dissociation Constants. A series of calibration solutions containing various [saccharide] was prepared in 0.1 M Phosphate-Buffered Saline (PBS). Each solutions contained 1 µM AS1 and they were adjusted to pH 7.4.

When a 1:1 saccharide-ligand complex is formed between saccahride and probe, one can describe the equilibrium as follows, where L and S represent probe and saccahride, respectively.

The total probe and saccahride concentration are defined as $[L]_0 = [L] + [LS]$ and $[S]_0 = [S] + [LS]$, respectively. With $[L]_0$ and $[S]_0$, the value of K_d is given by:

$$[LS]^2 - \left([L]_0 + [S]_0 + K_d\right)[LS] + [L]_0[S]_0 = 0,$$

$$[LS] = \frac{\left([L]_0 + [S]_0 + K_d\right) - \sqrt{\left([L]_0 + [S]_0 + K_d\right)^2 - 4[L]_0[S]_0}}{2} \qquad (1)$$

or

$$\left(F - F_{min}\right) = \left(\frac{\left([L]_0 + [S]_0 + K_d\right) - \sqrt{\left([L]_0 + [S]_0 + K_d\right)^2 - 4[L]_0[S]_0}}{2[L]_0}\right)\left(F_{max} - F_{min}\right) \qquad (2)$$

where F is the observed fluorescence intensity, F_{min} is the minimum fluorescence intensity, and F_{max} is the maximum fluorescence intensity. The K_d value that best fits the titration curve (Figures 1, S4, and S5) with Eq 2 was calculated by using the Excel program as reported.[8]

In order to determine the K_d^{TP} for the two-photon process, the TPEF spectra were obtained with a DM IRE2 Microscope (Leica) using the $xy\lambda$ mode at 800 Hz scan speed. They were excited by a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1580 mW, which corresponded to approximately 10 mW average power in the focal plane. The TPEF titration curves (Figures 1, S4, and S5) were obtained and fitted to Eq 2 (Figure 1b).
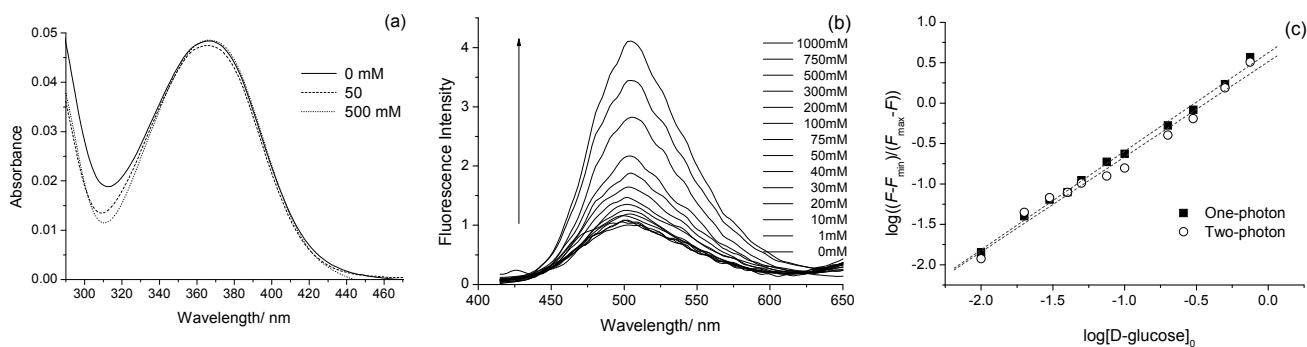

Figure S4. (a) One-photon absorption and (b) two-photon emission spectra of AS1 (PBS, pH 7.4) in the presence of D-glucose (0–1.0 M). (c) Hill plots for the complexation of AS1 with D-glucose (0–1.0 M). The excitation wavelengths for one- and two-photon processes were 365 and 780 nm, respectively.
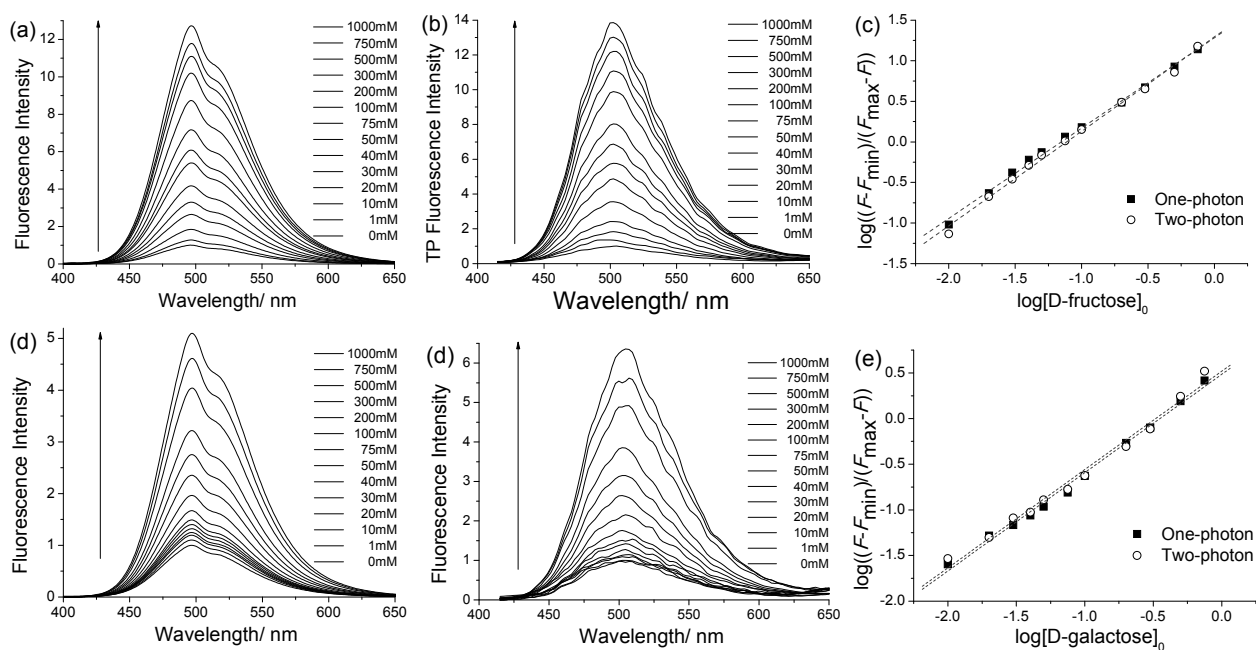
Figure S5. (a,d) One- and (b,e) two-photon emission spectra of AS1 (PBS, pH 7.4) in the presence of (a,b) D-fructose and (d,e) D-galactose (0–0.1 M). (c,f) Hill plots for the complexation of AS1 with (c) D-fructose and (f) D-galactose (0–0.1 M). The excitation wavelengths for one- and two-photon processes were 365 and 780 nm, respectively.

Measurement of Two-Photon Cross Section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[9] AS1 (5.0 µM) and D-glucose (0.75 M) were dissolved in Phosphate-Buffered Saline (PBS) (pH 7.4) and the two-photon excited fluorescence intensity was measured at 740–940 nm by using fluorescein (8.0×10^{-5} M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature.[10] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts s and r stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as S. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the TPA cross section of the reference molecule. The result is shown in Figure S6a. Moreover, the output intensity of TPEF was linearly dependent on the square of the input laser intensity, thereby confirming the occurrence of TPA (Figure S6b).
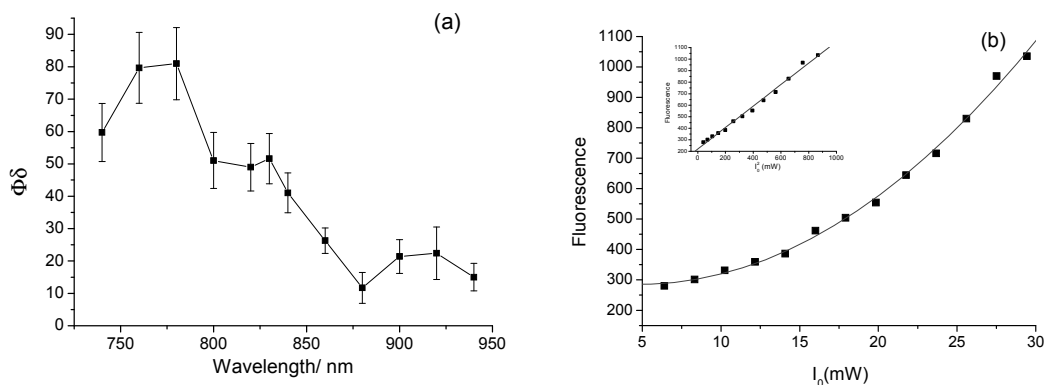
Figure S6. (a) Two-photon action spectra of 3 μM AS1 in the presence of D-glucose (0.75 M) in PBS buffer and (b) dependence of output fluorescence intensity (I_{out}) of 5 μM AS1 in PBS buffer on the input laser power (I_{in}). The insert shows the linear dependence of I_{out} on I_{in}^2 (780 nm, 90 MHz, τ = 160 fs).

HeLa Cell. HeLa human cervical carcinoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL). Two days before imaging, the cells were passed and plated on glass-bottomed dishes (MatTek). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. For labeling, the growth medium was removed and replaced with DMEM without FBS. The cells were treated and incubated with 2 μL of 1 mM AS1 in DMSO stock solution (2 μM AS1) at 37 °C under 5 % CO_2 for 30 min. The cells were washed three times with phosphate buffered saline (PBS; Gibco) and then imaged after further incubation in colorless serum-free media for 15 min.

Primary Cortical Neuronal Cells. Cortical neurons were prepared from cerebral cortices of one-day-old rats (Sprague–Dawley; SD). Cerebral cortices were dissociated in Hank's balanced salt solution (Gibco BRL, Gaithersburg, MD, USA), containing papain (1.5 unit/mL; Worthington Biochemical Corporation, NJ, USA). The cortical cells were gently triturated with a large-pore Pasteur pipette 3 to 4 times, and dissociated into individual cells using a small-pore Pasteur pipette. Single cortical neurons were plated onto 35 mm diameter plastic Petri dishes, which had been precoated with mixed poly-D-lysine (100 μg/ml) and laminin (4 μg/ml). The plating medium was neurobasal[TM] medium (Gibco) supplemented with B27 supplement (Gibco), 2 mM glutamine (Gibco), and penicillin/streptomycin (Gibco). Cultures were maintained at 36 °C in a humidified atmosphere of 5 % CO_2 incubator. The growth medium was identical to plating medium lacking fatal bovine serum and replenished twice weekly. The primary cortical neuronal cells were labeled with 2 μM AS1 and imaged by the same procedure as described above.

Two-Photon Fluorescence Microscopy. Two-photon fluorescence microscopy images of probe-labeled HeLa cells, primary cortical neuronal cells, and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×10 (NA = 0.30 DRY) and ×100 (NA = 1.30 OIL) objective lens. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1580 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 pixels at 400 Hz scan speed.
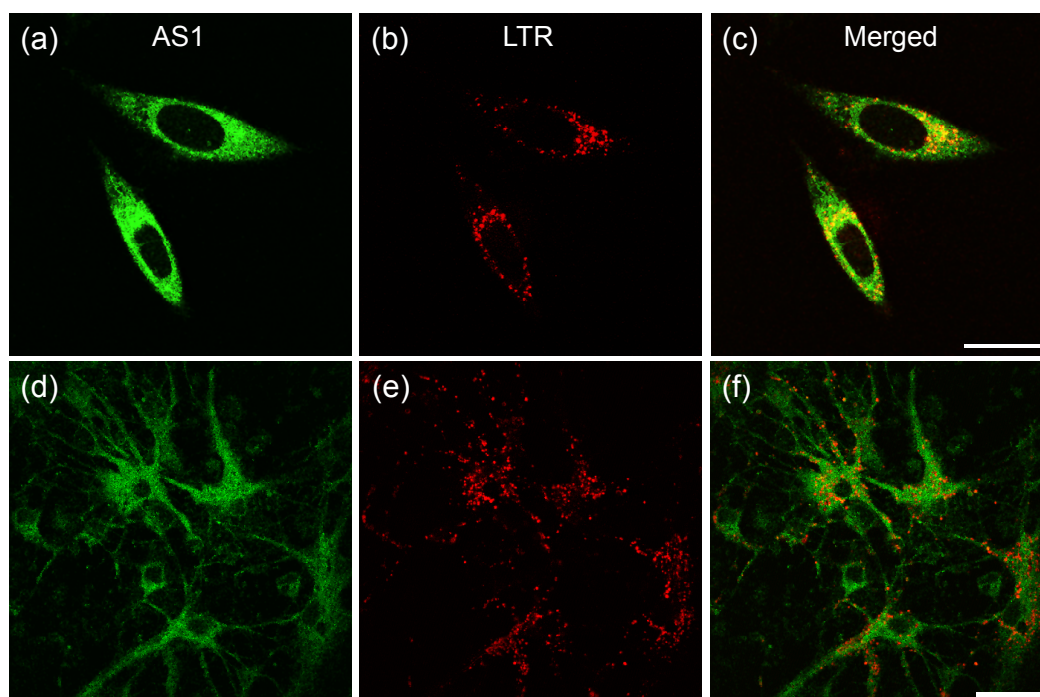

Figure S7. (a,d) TPM and (b,e) OPM images of HeLa (a-c) and primary cortical neuronal cells (d-e) colabeled with AS1 and LTR. (c,f) Merged images. The TPM images were collected at collected at 500-620 nm upon excitation at 780 nm with fs pulse. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 μm.
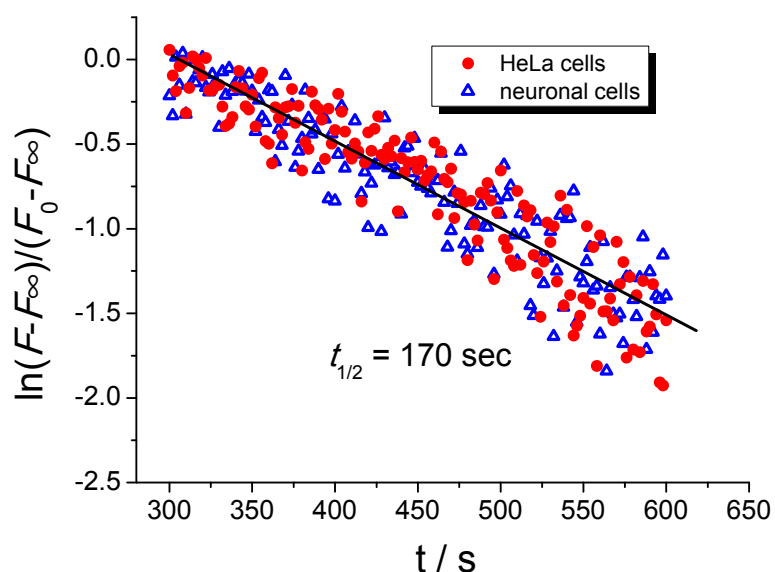
Figure S8. Decay rates of TPEF of 2 µM AS1-labeled HeLa cells and primary cortical neuronal cells, after addition of 20 mM D-glucose (Figure 2c). The thick lines are experimental data and the thin lines are first-order plots.
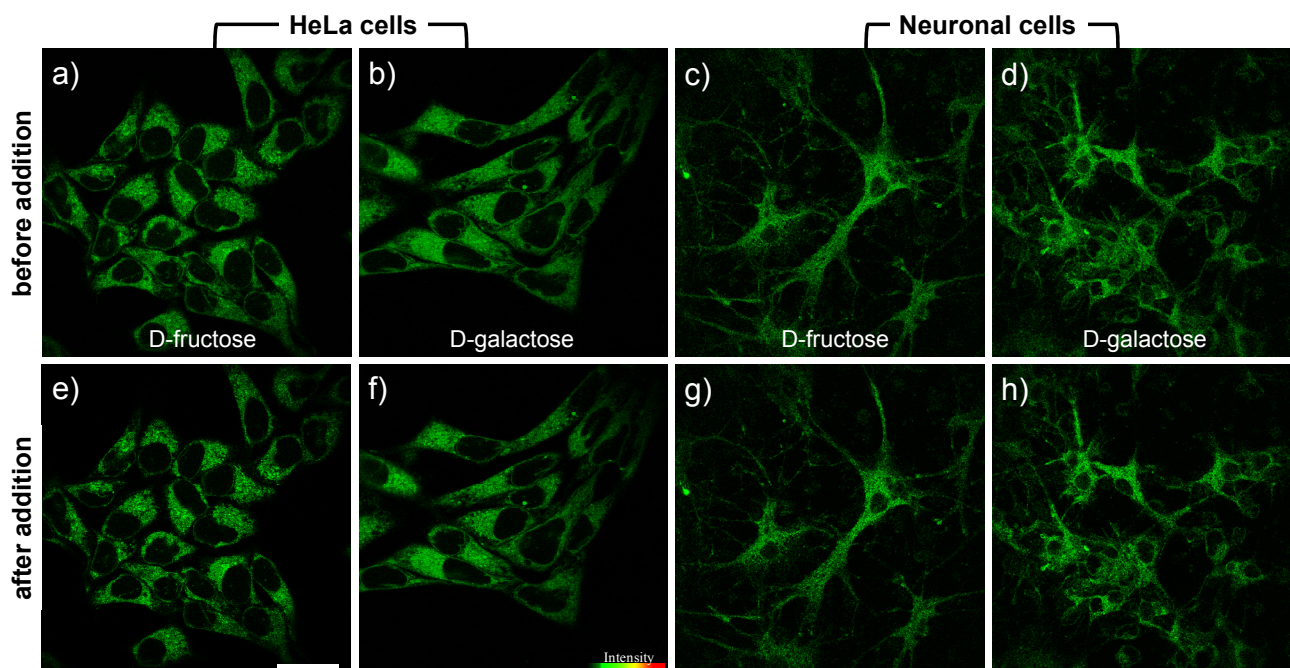

Figure S9. Pseudocolored TPM images of 2 µM AS1-labeled (a,b,e,f) HeLa cells and (c,d,g,h) primary cortical neuronal cells before and after addition of 20 mM (a,c,e,g) D-fructose and (b,d,f,h) D-galactose, respectively. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulse. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 µm.

Photostability. Photostability of AS1 was determined by monitoring the changes in TPEF intensity with time in the AS1-labeled HeLa cells chosen without bias. The TPEF intensity remained nearly the same for 1 hr, indicating high photostability.
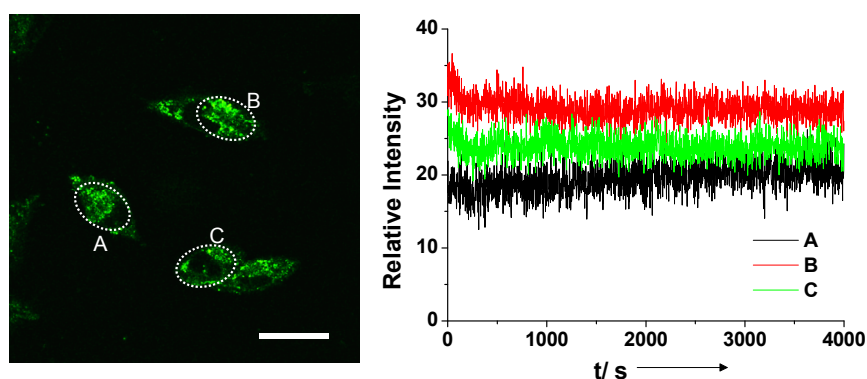

Figure S10. (*left*) TPM image of AS1-labeled HeLa cells treated with glucose. (*right*) Relative TPEF intensity measured at A−C in the *left* panel as a function of time. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulse. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 µm.
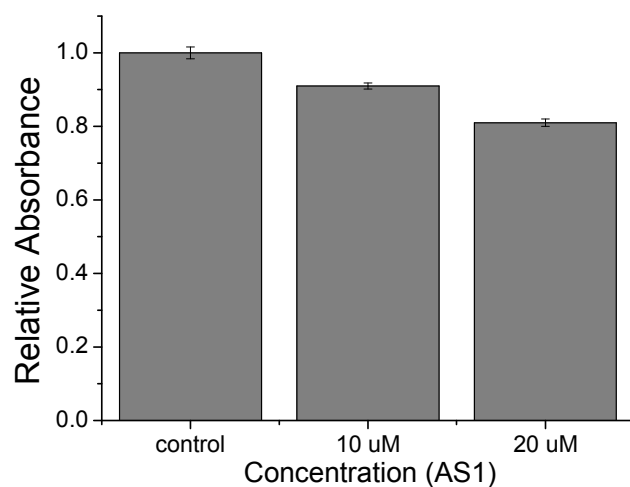

Figure S11. Viability of HeLa cells in the presence of AS1 as measured by using CCK-8 kit.

Preparation and Staining of a fresh Hippocampal slices. Slices were prepared from the hippocampi of 2-day-old rat (Sprague-Dawley; SD). Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; composition in mM: 138.6 NaCl, 3.5 KCl, 21 $NaHCO_3$, 0.6 NaH_2PO_4, 9.9 D-glucose, 1 $CaCl_2$, and 3 $MgCl_2$). Slices were incubated with 20 μM AS1 in ACSF bubbled with 95% O_2 and 5% CO_2 for 30-40 min at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope.
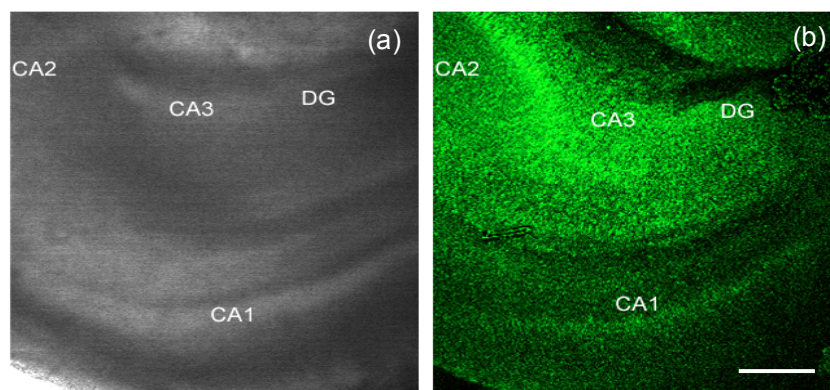

Figure S12. Images of a fresh rat hippocampal slice stained with 20 μM AS1. a) Bright field image shows the CA1 and CA3 regions as well as the dentate gyrus (DG) upon magnification 10×. b) 30 TPM images were accumulated along the z-direction at the depth of ~100-200 μm with magnification 10× to visualize the average distribution of the saccharide in the same regions. Scale bar, 300 μm.
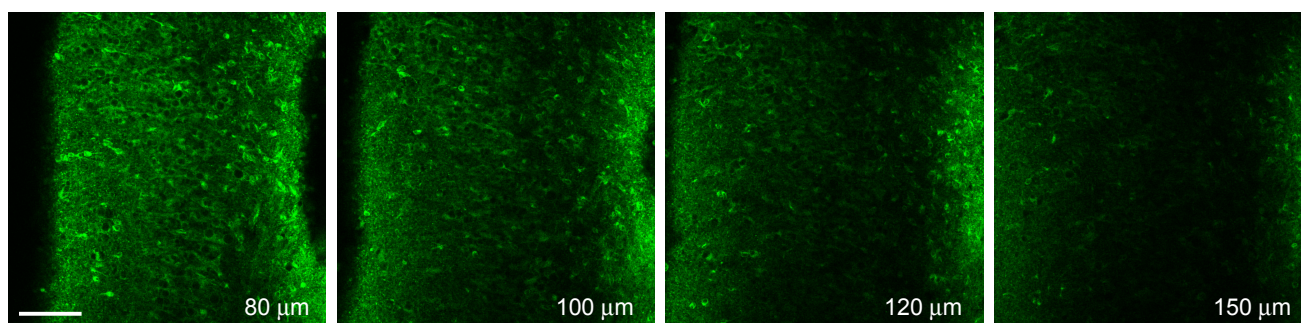

Figure S13. TPM images show the CA1 region by magnification at 20× of a fresh rat hippocampal slice stained with 20 μM AS1. The TPEF images were collected at 500-620 nm upon excitation at 780 nm with fs pulses. Scale bar, 150 μm.
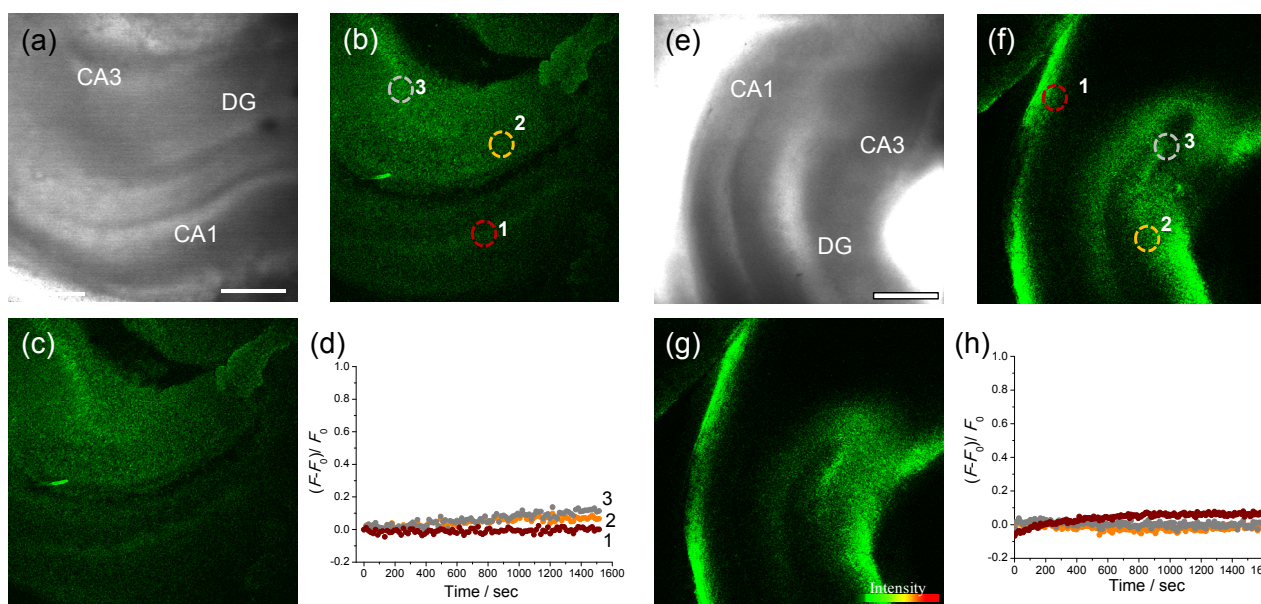
Figure S14. Images of a fresh rat hippocampal slice stained with 20 μM AS1. (a,e) Bright field images. (b,c,f,g) TPM images obtained at a depth of ca. 100 μm with magnification 10× (b,f) before and (c,g) 1000 sec after treatment of 50 nM insulin, 50 mM KCl, and 50 mM D-fructose (b,c) and D-galactose (f,g), respectively. (d,h) Time courses of TPEF intensity at designated positions 1-3 in b and f. The TPEF were collected at 500−620 nm upon excitation at 780 nm with a femtosecond pulse. Scale bar, 300 μm.

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