< Supporting Information >

Impact of molecular charge on GLUT-specific cellular uptake of glucose bioprobes and *in vivo* application of the glucose bioprobe, GB2-Cy3

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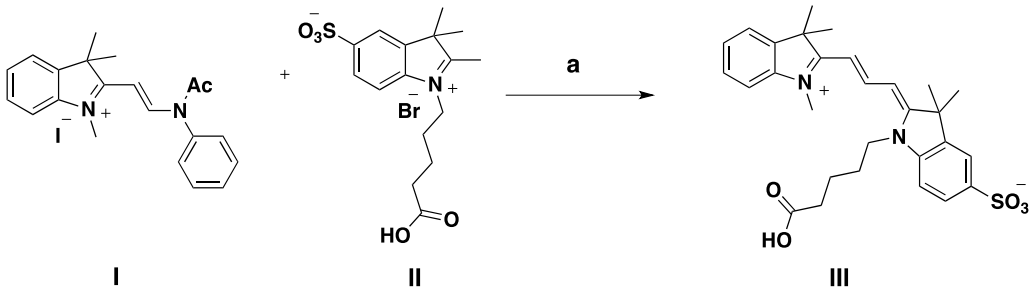
1. General synthetic information

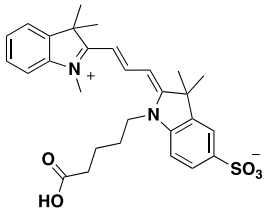
^{1}H NMR spectra were recorded on a Bruker DRX-300 [Bruker Biospin] and a Varian Inova-500 [Varian Assoc.], and chemical shifts were measured in ppm relative to internal tetramethylsilane (TMS) standard or specific solvent signal. Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); td (triplet of doublet); bs (broad singlet), etc. Coupling constants were reported in Hz. Low resolution mass spectrometry (LRMS) analyses were performed with Finnigan MSQ Plus Surveyer HPLC/MS system [Thermo Electron Corp., USA] using electron spray ionization (ESI). All reagents in this synthetic procedure were purchase from Sigma-Aldrich and TCI. The progress of reaction was monitored using thin-layer chromatography (TLC) (silica gel 60 F_{254} 0.25 mm), and components were visualized by observation under UV light (254 and 365 nm) or by treating the TLC plates with anisaldehyde staining solution followed by heating. Silica gel 60 (0.040–0.063 mm) used in flash column chromatography was purchased from Merck. All reactions were conducted in oven-dried glassware under dry argon atmosphere, unless otherwise specified. CH_2Cl_2 was distilled from CaH_2 immediately prior to use. Other solvents and organic reagents were purchased from commercial venders and used without further purification unless otherwise mentioned.

2. Synthesis of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2



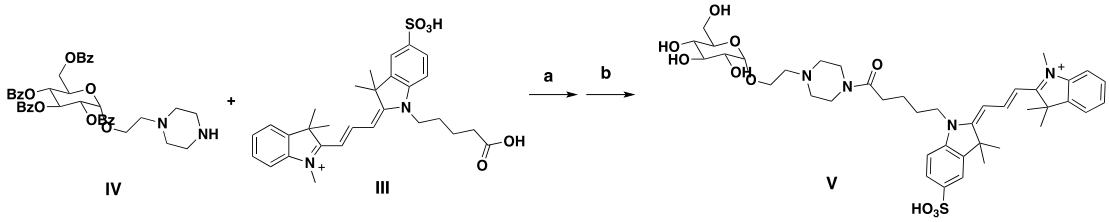
Scheme S1 Synthetic procedure of Cy3-S1. (a) pyridine, Ac_2O, 120°C, 2h, 17%

Cy3-S1 (III), *2-((1E,3E)-3-(1-(4-carboxybutyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium*



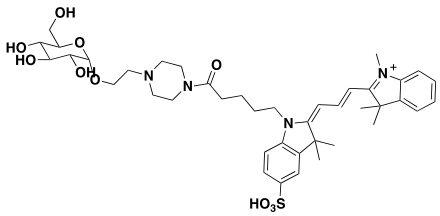
Intermediate **I** and **II** were synthesized as previously reported.[1,2] Intermediated **I** (100 mg, 0.22 mmol) and intermediated **II** (94 mg, 0.22 mmol) were dissolved in pyridine (5 mL) and Ac_2O (5 mL). The reaction mixture was stirred at 120°C for 2 h. The solvents were evaporated *in vacuo*, and remaining liquid was dissolved with brine and dichloromethane. The organic layer was separated and the aqueous layer was extracted three times with dichloromethane. The combined organic layer was dried over anhydrous Na_2SO_4(s) and filtered through a celite-packed glass filter. The filtrate was concentrated *in vacuo* and purified with HPLC to provide **Cy3-S1**, **III** (20 mg).

^{1}H-NMR (300 MHz, MeOD): δ 8.56 (t, *J* = 13.72 Hz, 1H), 7.98−7.83 (m, 2H), 7.61−7.53 (m, 1H), 7.51−7.29 (m, 4H), 6.56−6.39 (m, 2H), 4.22−4.10 (m, 2H), 3.76−3.69 (m, 3H), 3.68−3.60 (m, 1H), 2.49−2.36 (m, 2H), 1.78 (s, 6H), 1.77 (s, 6H), 1.29 (s, 3H); LRMS (ESI$^+$) m/z calcd for $C_{29}H_{35}N_2O_5S$ $[M]^+$: 523.23 Found : 523.08.



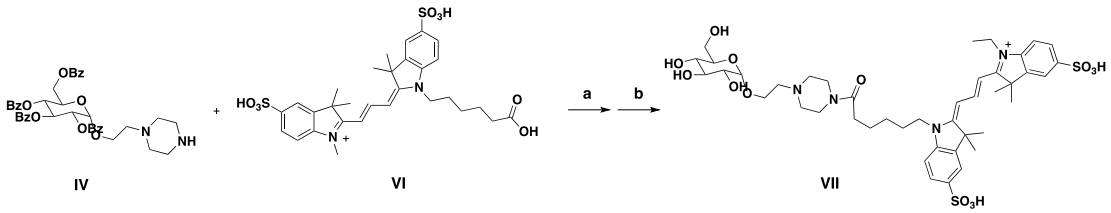
Scheme S2 Synthetic procedure of GB2-Cy3-S1. (a) TSTU, DIPEA, DMF, r.t., overnight; (b) NaOMe, MeOH, r.t., 3 h. 21% (two steps)

GB2-Cy3-S1 (V), *2-((1E,3E)-3-(3,3-dimethyl-1-(5-oxo-5-(4-(2-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)piperazin-1-yl)pentyl)-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium*



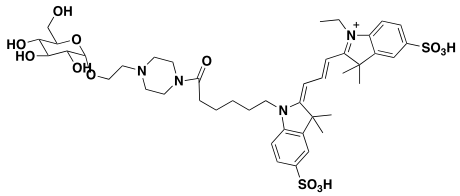
Intermediate **IV** was synthesized as previously reported.[3] **Cy3-S1** (**III**) (13.5 mg, 0.026 mmol) were dissolved in DMF (1 mL). DIPEA (13.5 μl, 0.077 mmol) and TSTU (11.6 mg, 0.039 mmol) were added to the reaction mixture and stirred at room temperature for 1 h. Then, intermediated IV and was added to the reaction mixture and stirred at room temperature for overnight. The solvents were evaporated *in vacuo*, and remaining liquid was dissolved with methanol (500 μl). Sodium methoxide (0.5 M solution in methanol, 154 μl, 0.077 mmol) was added to the reaction mixture and stirred at room temperature for 3 h. The solvents were evaporated *in vacuo*, and remaining NaOMe was quenched with 2 N HCl. The remaining liquid was concentrated *in vacuo* and purified with HPLC to provide **GB2-Cy3-S1**, **V** (4.3 mg).

^{1}H-NMR (300 MHz, D_2O): δ 8.54 (t, *J* = 13.45 Hz, 1H), 7.92−7.83 (m, 2H), 7.61 (d, *J* = 7.34 Hz, 1H), 7.55−7.47 (m, 1H), 7.44−7.38 (m, 2H), 7.35 (d, *J* = 8.31 Hz, 1H), 6.42 (d, *J* = 13.69 Hz, 1H), 6.33 (d, *J* = 13.21 Hz, 1H), 5.00 (d, *J* = 3.91 Hz, 1H), 4.16−4.05 (m, 4H), 4.02−3.93 (m, 1H), 3.91−3.82 (m, 4H), 3.81−3.62 (m, 11H), 3.53−3.39 (m, 2H), 2.54 (t, *J* = 7.09 Hz, 2H), 1.93-1.86 (m, 2H), 1.83−1.66 (m, 15H); LRMS (ESI$^-$) m/z calcd for $C_{41}H_{56}N_4O_{10}S$ $[M-H]^-$: 796.37 Found : 795.55.



Scheme S3 Synthetic procedure of GB2-Cy3-S2. (a) TSTU, DIPEA, DMF, r.t., overnight; (b) NaOMe, MeOH, r.t., 3 h. 45% (two steps).

GB2-Cy3-S2 (VII), *2-((1E,3E)-3-(3,3-dimethyl-1-(6-oxo-6-(4-(2-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)piperazin-1-yl)hexyl)-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indol-1-ium*



Intermediate **VI** was purchased from GE healthcare. Intermediate **VI** (7.5 mg, 0.011 mmol) were dissolved in DMF (1 mL). DIPEA (8.26 μl, 0.044 mmol) and TSTU (5.36 mg, 0.017 mmol) were added to the reaction mixture and stirred at room temperature for 1 h. Then, intermediated IV and was added to the reaction mixture and stirred at room temperature for overnight. The solvents were evaporated *in vacuo*, and remaining liquid was dissolved with methanol (400 μl). Sodium methoxide (0.5 M solution in methanol, 100 μl, 0.033 mmol) was added to the reaction mixture and stirred at room temperature for 2 h. The solvents were evaporated *in vacuo*, and remaining NaOMe was quenched with 2 N HCl. The remaining liquid was concentrated *in vacuo* and purified with HPLC to provide **GB2-Cy3-S2**, **VII** (4.8 mg).

^{1}H-NMR (500 MHz, D_2O): δ 8.48 (t, *J* = 13.45 Hz, 1H), 7.89 (d, *J* = 0.98 Hz, 2H), 7.83 (ddd, *J* = 8.31, 4.40, 1.47 Hz, 2H), 7.33 (t, *J* = 8.07 Hz, 2H), 6.35 (dd, *J* = 16.63, 13.69 Hz, 2H), 5.01 (d, *J* = 3.91 Hz, 1H), 4.14−4.01 (m, 6H), 3.92−3.83 (m, 3H), 3.81−3.70 (m, 3H), 3.69−3.60 (m, 3H), 3.59−3.41 (m, 5H), 2.43 (t, *J* = 7.34 Hz, 2H), 1.87−1.77 (m, 3H), 1.70 (s, 6H), 1.69 (s, 6H), 1.63−1.53 (m, 3H), 1.42−1.30 (m, 5H); LRMS (ESI$^+$) m/z calcd for $C_{43}H_{61}N_4O_{13}S_2$ $[M]^+$: 905.37 Found : 905.43.

GB2-Cy3

Synthesis of GB2-Cy3 was followed as previous reported.[3]

3. Optical properties of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2

20 μM of each compound was dissolved in methanol and water and subjected for measuring fluorescence intensity and absolute quantum yield. Fluorescence emission spectra were measured by Cary Eclipse Fluorescence spectrophotometer [Varian Assoc.]. Absolute quantum yield was measured by absolute PL quantum yield measurement system QE-1000 [OTSUKA Electronics].

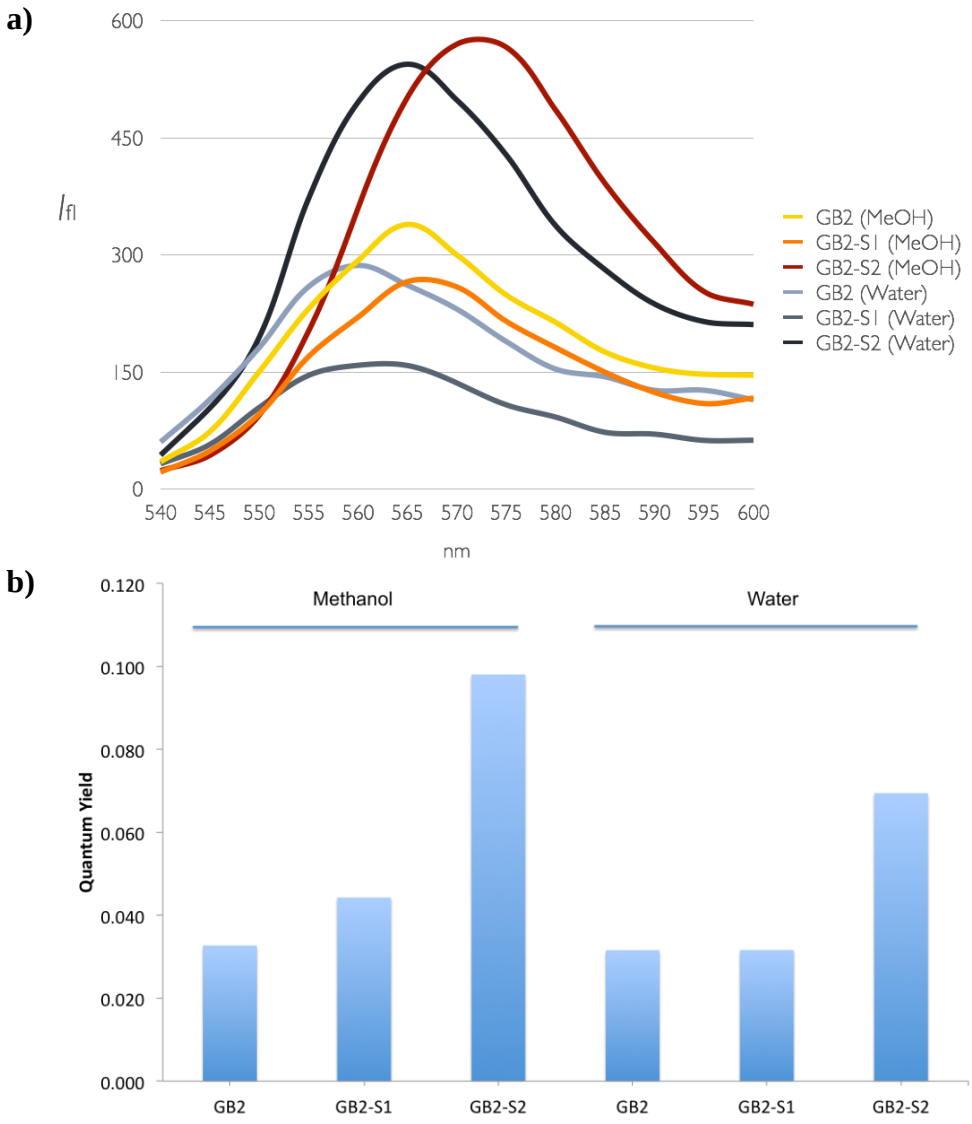


Fig. S1 Fluorescence emission spectra (a) and absolute quantum yields (b) of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2 in methanol and water

4. Cellular uptake of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2

Cell culture

HeLa cells were obtained from American Type Culture Collection [ATCC]. HeLa human cervical cancer cell lines were cultured in RPMI 1640 [GIBCO, Invitrogen] supplemented with heat-inactivated 10 % (v/v) fetal bovine serum [GIBCO, Invitrogen] and 1 % (v/v) antibiotic-antimycotic solution [GIBCO, Invitrogen]. Cells were maintained in a humidified atmosphere of 5 % CO2 incubator at 37 °C, and cultured in 100-mm cell culture dish [CORNING].

Fluorescence cellular imaging of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2

Hela cells were cultured on glass-bottom dishes [Corning] for 24 h. The cells were treated with the various concentrations of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2. The final concentration of GB2 was adjusted to 10 μM. After 30 min incubation at 37 °C, the cells were washed twice with PBS and prepared for imaging by adding 1 mL of PBS. Fluorescence images were obtained at an excitation wavelength of 540 nm using an Olympus inverted microscope (IX71), equipped for epi-illumination with a halogen bulb [Philips]. The emission signal of each experiment was detected using the following spectral settings: a 510–550 nm band pass exciter filter, 570 nm center wavelength chromatic beam splitter, and a 590 nm long pass barrier filter [Olympus filter set, U-MWG2]. The emission signals were detected with a 12.5 megapixel recording digital color camera [DP71; Olympus]. The images were analyzed using the Image-Pro Plus® 6.2 program, and bar graphs were processed using GraphPad Prism 5. The quantified data are the mean of fluorescent signal measurements of 40–50 cells from at least 3 independent experiments.

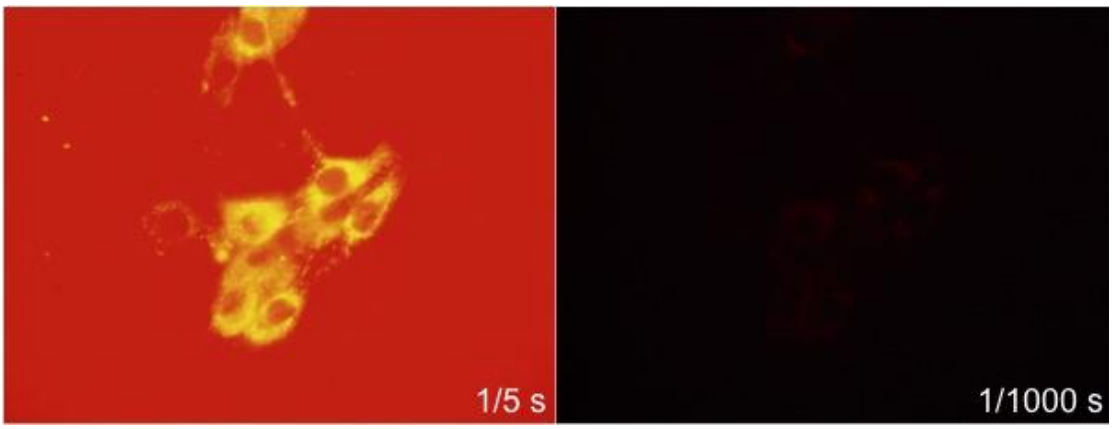


Fig. S2 Fluorescence microscopic images after cellular uptake of GB2-Cy3 (10 μM) in HeLa cells. The images were captured after 1/1000 and 1/5 sec exposure time.

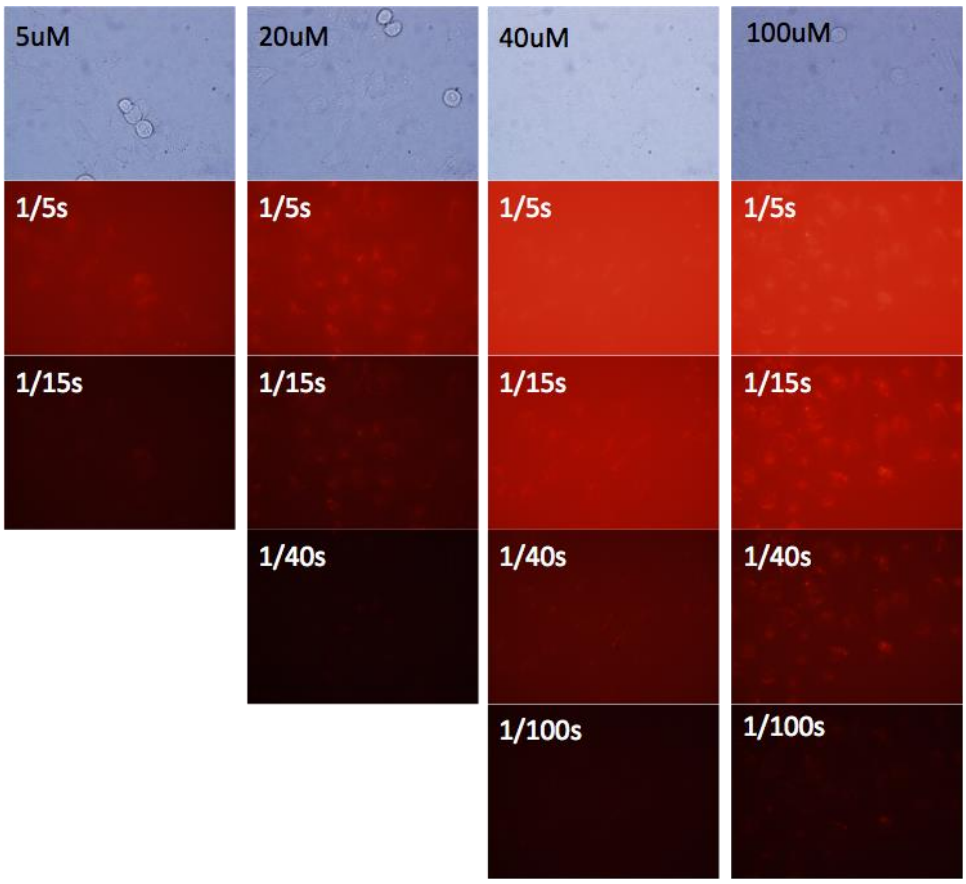


Fig. S3 Fluorescence microscopic images after cellular uptake of GB2-Cy3-S2 with various concentrations in HeLa cells. The images were captured after exposure time from 1/5 sec to 1/100 sec.

5. Glucose competition assay

To measure the competitive cellular uptake of GB2-Cy3 and GB2-Cy3-S1, HeLa cells cultured on a cover glass bottom dish were incubated for 30 min at 37 °C in the presence of individual fluorescent glucose bioprobes, GB2-Cy3 and GB2-Cy3-S1 in DMEM containing 55 mM D-glucose, or no glucose. The final concentration of fluorescent glucose analogs was adjusted to 10 μM. Exposure time for GB2-Cy3 and GB2-Cy3-S1 uptake imaging is 1/1000 sec and 1/15 sec. After washing with cold PBS, fluorescence images were obtained at an excitation wavelength of 540 nm using a fluorescence microscope (Olympus IX71).

6. Molecular charges and GLUT-specific uptake properties of the previously reported glucose bioprobes

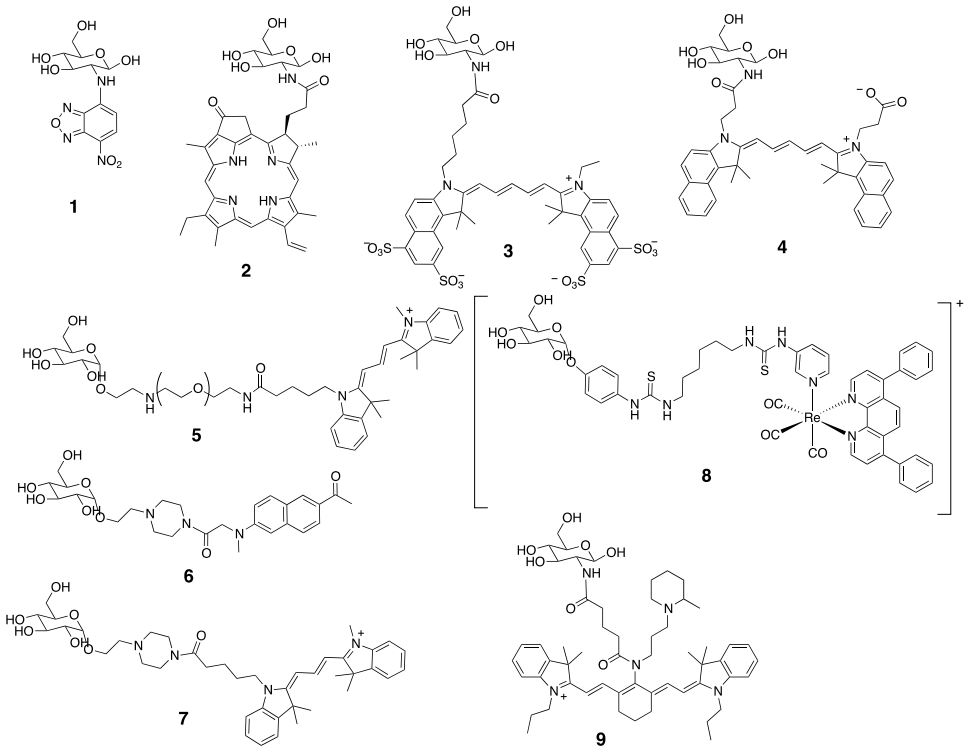


Fig. S4 Chemical structures of the previously reported glucose bioprobes.

Structure of previously reported glucose bioprobes (Fig. S3) and its biophysical properties (Table S1) supports our hypothesis of the manuscript. Cellular uptake of neutral and cationic charged glucose bioprobes are GLUT-specific as previously reported, but that of anionic and zwitterionic charged glucose bioprobes are not.

Table S1 Molecular charges and biophysical properties of the previously reported glucose bioprobes.

Glucose Bioprobe	Expected Charge	Calculated Charge	GLUT specific cellular uptake	Reference number in the Manuscript
1	Neutral	−0.02	Specific	5
2	Neutral	0.39	Specific	6
3	Anion	−2.82	Nonspecific	7
4	Zwitterion	0.63	Nonspecific	8
5	Cation	1.39	Specific	9
6	Neutral	−0.05	Specific	10
7	Cation	1.39	Specific	11
8	Cation	2.77	Specific	12
9	Cation	2.94	Specific	13

7. Measurement of GB2-Cy3 uptake in zebrafish

Zebrafish Maintenance

Zebrafish were maintained in accordance with standard guidelines.[4] Care and treatment of zebrafish were conducted in accordance with guidelines established by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology, Republic of Korea. Wild-type zebrafish (*Danio rerio*) were maintained in 10 L glass tanks with 28.5 °C filtered tap water and a photoperiod of 14:10 h (light:dark). Fish were fed twice daily using combination of brine shrimp (*Artemia salina*) and dry food (Amazon Flake). For all experiments, embryos were obtained by *in vitro* fertilization following standard procedures.[5]

Evaluation of GB2-Cy3 uptake in zebrafish

Fish eggs were incubated in E3 water (5 mM NaCl, 0.17 mM KCl, 033 mM $CaCl_2$ and 0.33 mM $MgSO_4$ in distilled water) supplemented with 0.2 mM 2-phenylthiourea (a depigmentation compound that increases larvae transparency). At 72 hpf the larvae were placed into a 96-well plate at a density of 6 embryos/well for microplate reader analysis, or 3 embryos/well for fluorescence microscopy, in 200 μL E3 water. Larvae were incubated with GB2-Cy3 for 3 h. To assess the effect of test compounds on GB2-Cy3 uptake, the larvae were exposed to the compounds for 1 h prior to the addition of GB2-Cy3. E3 water containing the test compounds were then removed and replaced with GB2-Cy3 dissolved in E3 water.

For fluorescence microscopy, the E3 water containing GB2-Cy3 was removed and the larvae were then washed with E3 water and anesthetized with 0.02 % tricaine dissolved in E3 water. The larvae were then placed on glass microscope slides in plastic chambers (1.6 cm diameter and 3 mm depth) containing 3% methylcellulose dissolved in E3 water. Images of GB2-Cy3 uptake were captured using fluorescent microscopy (DM2500, Leica) equipped with a digital camera (DFC425C, Leica). Three larvae were imaged for each experimental sample. Images were processed using the Lieca Application Suite software and Photoshop CS4 (Adobe Systems Incorporated). GB2-Cy3 uptake into the larvae was quantified with Image J software (National Institutes of Health).

To measure GB2-Cy3 uptake using a fluorescent microplate reader, larvae from each treatment group were placed in a 1.5 mL microfuge tube and lysed with 120 μL CelLytic M solution (Sigma-Aldrich) and mechanical disaggregation using watchmaker forceps. Larvae were then sonicated (Vibra-Cell VCX500) at 4 ℃ with a 10 sec 'on' and 5 sec 'off' pulse for 15 min (i.e. total time of 10 min for sonication plus 5 min pause), followed by centrifugation at 10000 rpm for 10 min. The supernatant (100 μL) was transferred to a 96-well plate and NBDG signal was measured with a fluorescent microplate reader (SpectraMAX Gemini XS, λ_{ex}=485 nm, λ_{em}=535 nm). For microplate reader analysis, each

experimental sample was measured in triplicate wells. A schematic of the protocol to assess glucose homeostasis in zebrafish using GB2-cy3 is shown below:

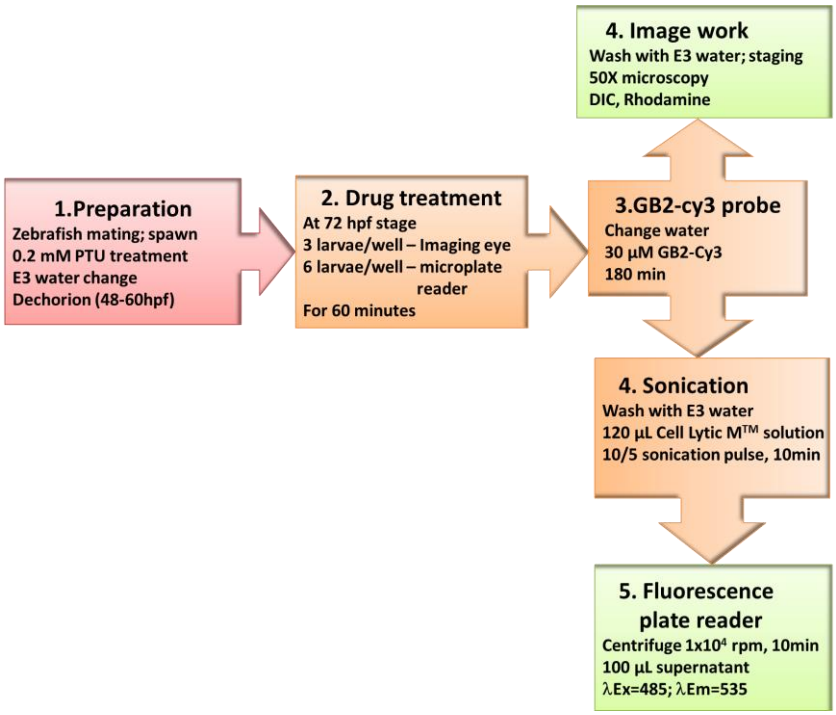


Fig. S5 Protocol of zebrafish-based screening systems for *in vivo* glucose uptake using GB2-Cy3

Statistical analysis

The student's *t*-test was used for comparison between experimental groups (Microsoft Excel, 2010 version). *P* values of <0.05 were considered significant. Unless otherwise stated, all results are the average of three independent experiments and the error bars are standard deviation of the mean.

Reagents

4,6-O-ethylidene-α-D-glucose (4,6-EDG), insulin from bovine pancreas and rosiglitazone were purchased from Sigma-Aldrich. 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was purchased from Invitrogen. Cytochalasin B was purchased from Santa Cruz Biotechnology. Emodin was a gift from Professor Wong-Keun Oh, Seoul National University, Republic of Korea. GAPDS was a gift from Professor Young-Tae Chang, National University of Singapore.

8. Time-dependent uptake of GB2-Cy3 in zebrafish

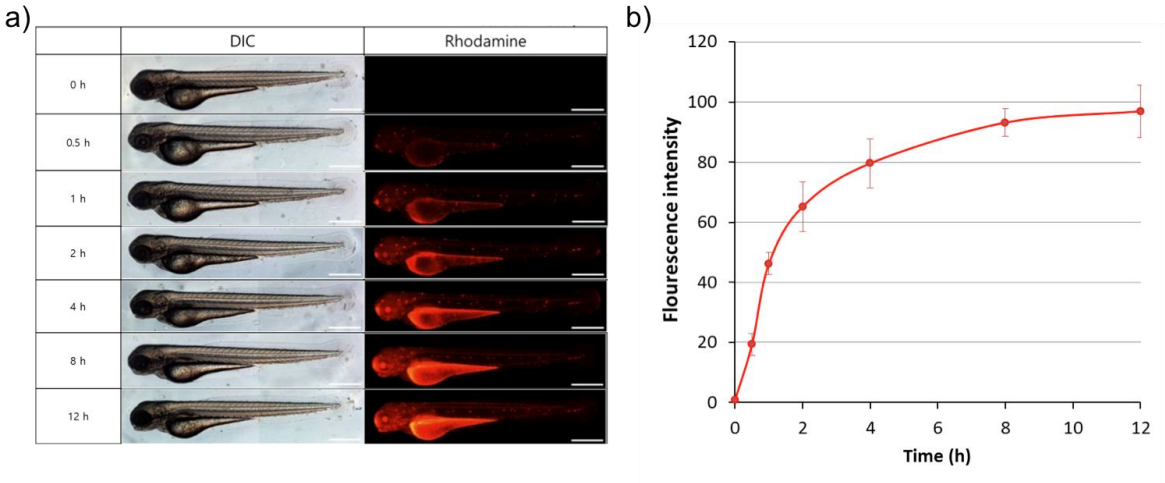


Fig. S6 Time-dependent uptake of GB2-Cy3 in zebrafish. Fluorescence intensity of GB2-Cy3 in zebrafish larval eye was dramatically increased within 2 h and saturated after 12 h upon treatment of GB2-Cy3 (30 μM) in E3 water. On the basis of this result, we concluded that 3 h incubation is optimum condition for GB2-Cy3 zebrafish imaging. The scale bar represents 500 μm.

9. Dose-dependent uptake of GB2-Cy3 in the zebrafish larval eye

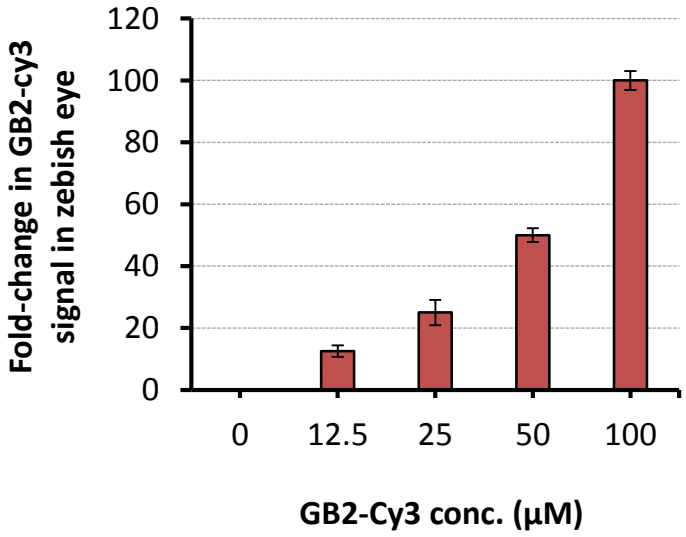


Fig. S7 Dose-dependent uptake of GB2-Cy3 in the zebrafish larval eye via the image-based measurement of fluorescence intensity. This uptake pattern is highly correlated with GB2-Cy3 uptake measured by the fluorescence intensity in zebrafish lysate using microplate reader.

10. Increase of GB2-Cy3 uptake in zebrafish upon treatment with glucose uptake enhancers (insulin, GAPDS, and emodin)

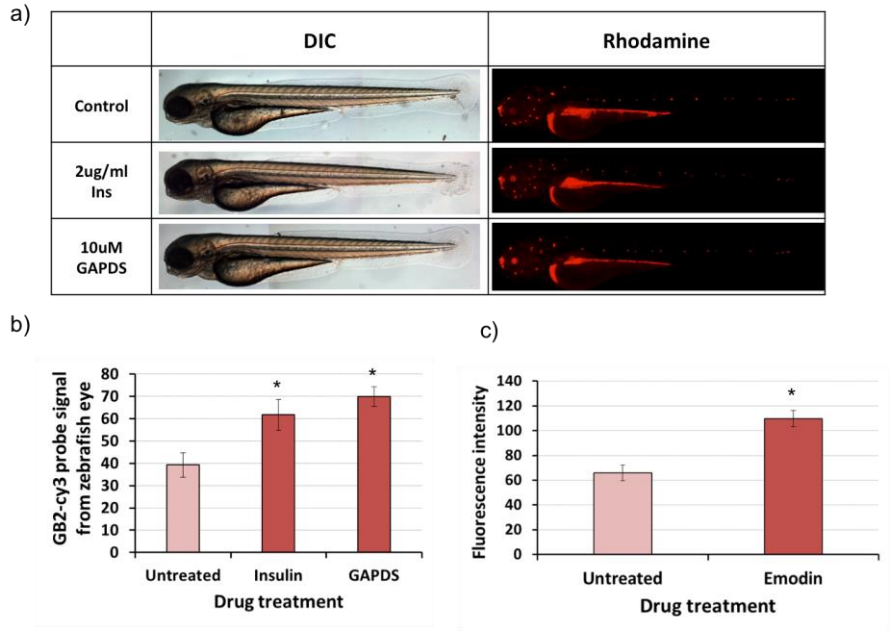


Fig. S8 Glucose uptake enhancers (insulin, GAPDS, and emodin) increased GB2-Cy3 uptake in zebrafish. 2.5 µg/mL concentration of emodin was used for experiment.

11. Analysis of GB2-Cy3 uptake in zebrafish larval tissues

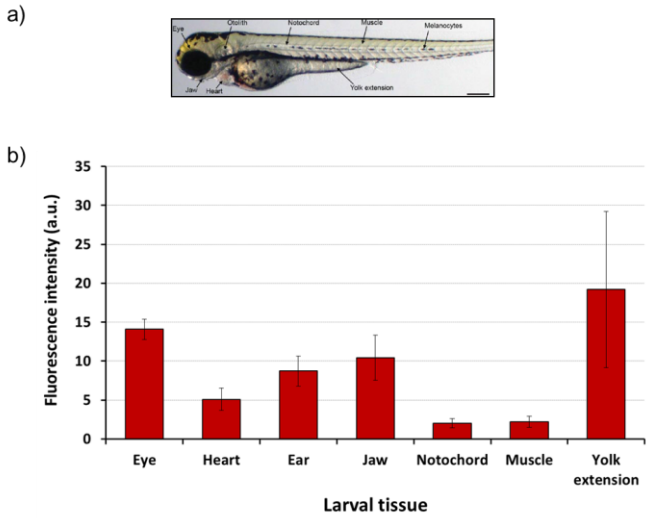


Fig. S9 Assessment of GB2-Cy3 uptake in different larval tissues. (a) Seven tissues were analyzed in 72 hpf larvae. Scale bar = 240 µm. (b) It was observed that the eye showed relatively high GB2-Cy3 uptake with relatively low variation between larvae. Error bar is the standard deviation (n=6). Please note that melanocytes could not be measured in this study, because larvae pigmentation was reduced by treatment with 2-phenylthiourea (as described in section 7 of the supporting information).

12. References

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