SUPPORTING INFORMATION

Turn-on Fluorescent Glucose Transport Bioprobe Enables Wash-Free Real-Time Monitoring of Glucose Uptake Activity in Live Cells and Small Organism

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1. Supporting Figures

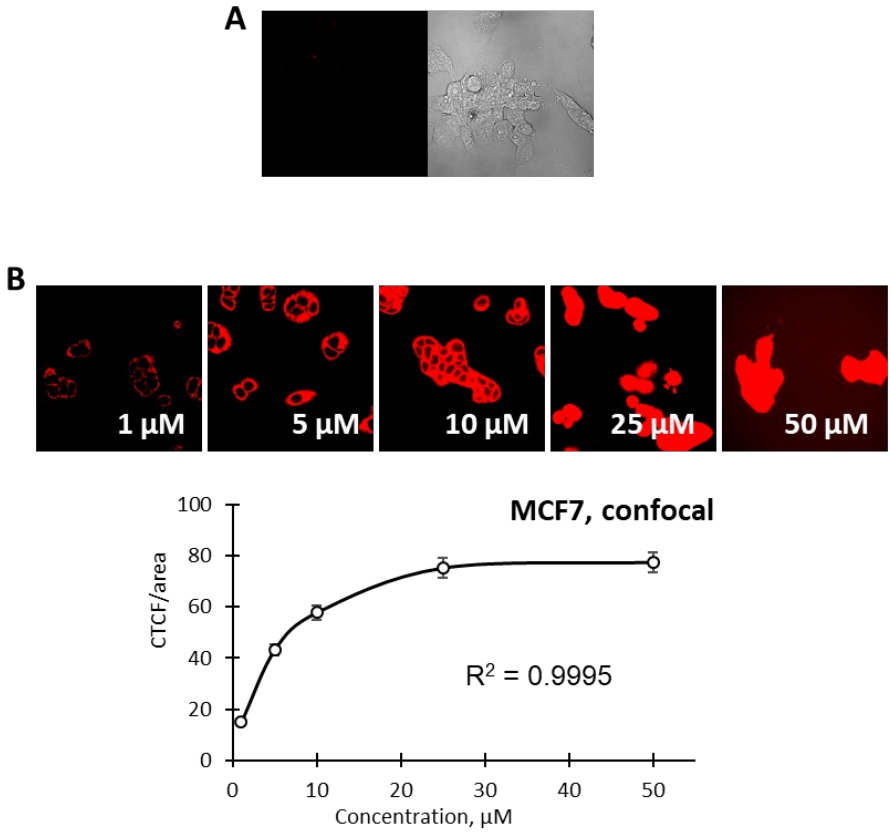

Figure S1. GluRho exhibits concentration-dependent uptake. A) Fluorescence images of probe-treated MCF7 cells after 15 min incubation at 37 °C. Confocal images obtained using Olympus confocal microscope, 60X objective, exc. 559 nm / em. 603 nm at identical imaging parameters. The differences in probe uptake at different incubation and post-incubation times were derived through quantification of fluorescence images using ImageJ as CTCF/Area: CTCF = (Integrated Density − (Area of selected cell × Mean fluorescence of background readings))/area of the selected cell. Error bars represent the standard deviation in fluorescence between 15-20 cells. Solid curve represents the curve fitting. Curve fitting were carried out using GraphPad Prism 10. The optimal fitting was obtained with non-linear regression analysis (Y=Bmax*X/(Kd + X)).

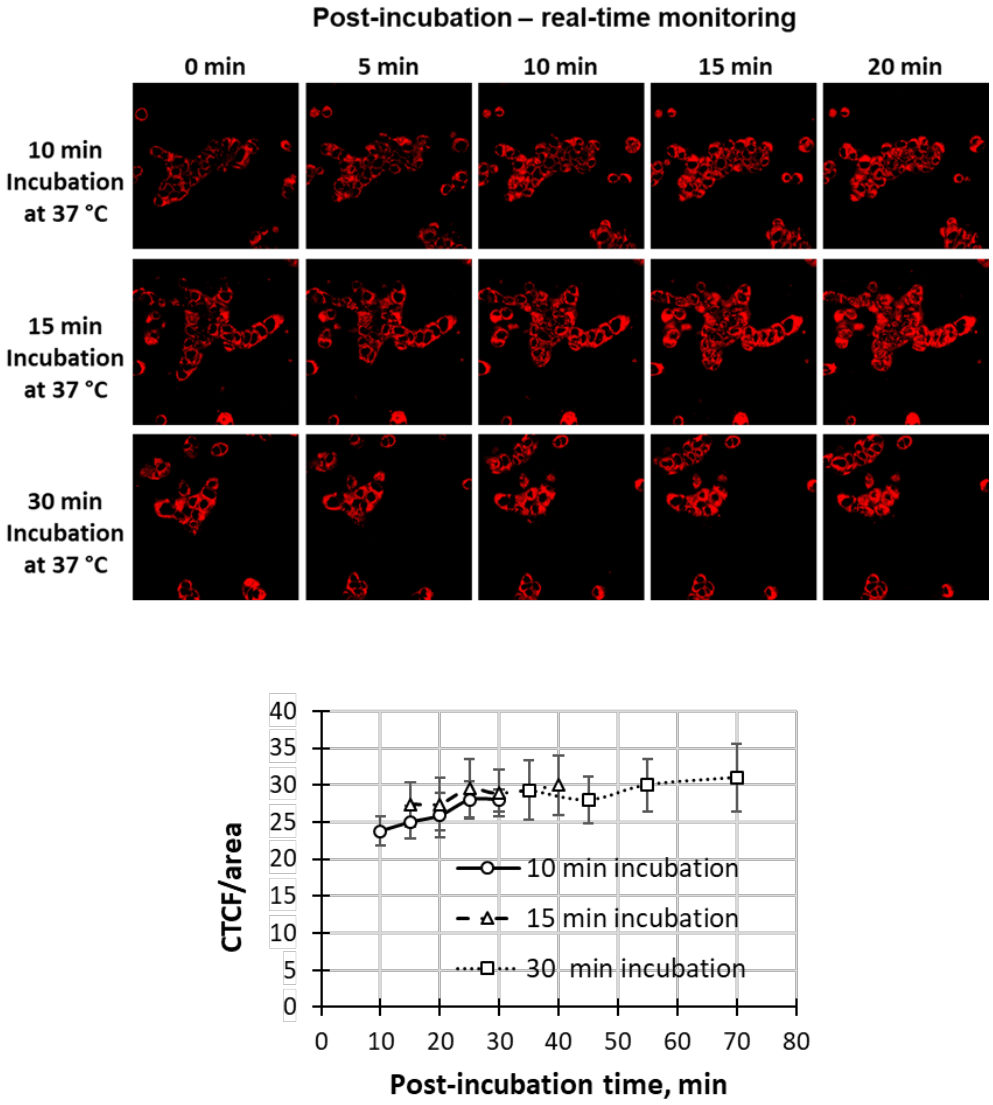

Figure S2. Stability of probe fluorescence in cells at different incubation times. Fluorescence images were taken for MCF7 cells pretreated with 2 μM probe after different incubation (37 °C) and post-incubation (room temperature) times. Confocal images obtained using Olympus confocal microscope, 60X objective, exc. 559 nm / em. 603 nm at identical imaging parameters. The differences in probe uptake at different incubation and post-incubation times were derived through quantification of fluorescence images using ImageJ as CTCF/Area: CTCF = (Integrated Density − (Area of selected cell × Mean fluorescence of background readings))/area of the selected cell. Error bars represent the standard deviation in fluorescence between 15-20 cells.

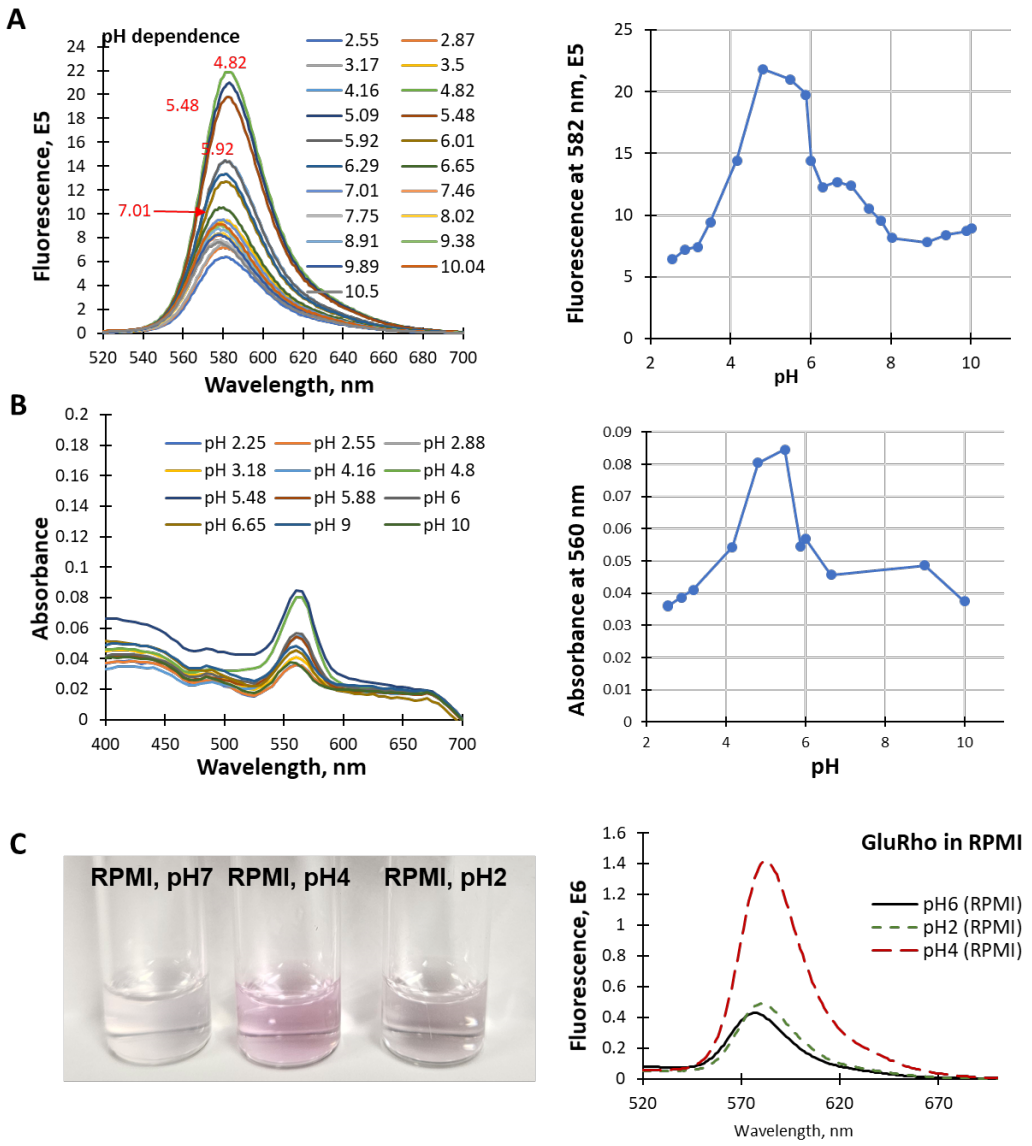

Figure S3. Impact of pH on GluRho (10 μM) fluorescence (**A**) and absorbance (**B**). (A) and (B) data obtained in respective aqueous buffers at 500 nm excitation. **C**) Effect of pH on GluRho (10 μM) in a dye-free RPMI culture media. GluRho was used from the 10 mM stock solution in DMSO.

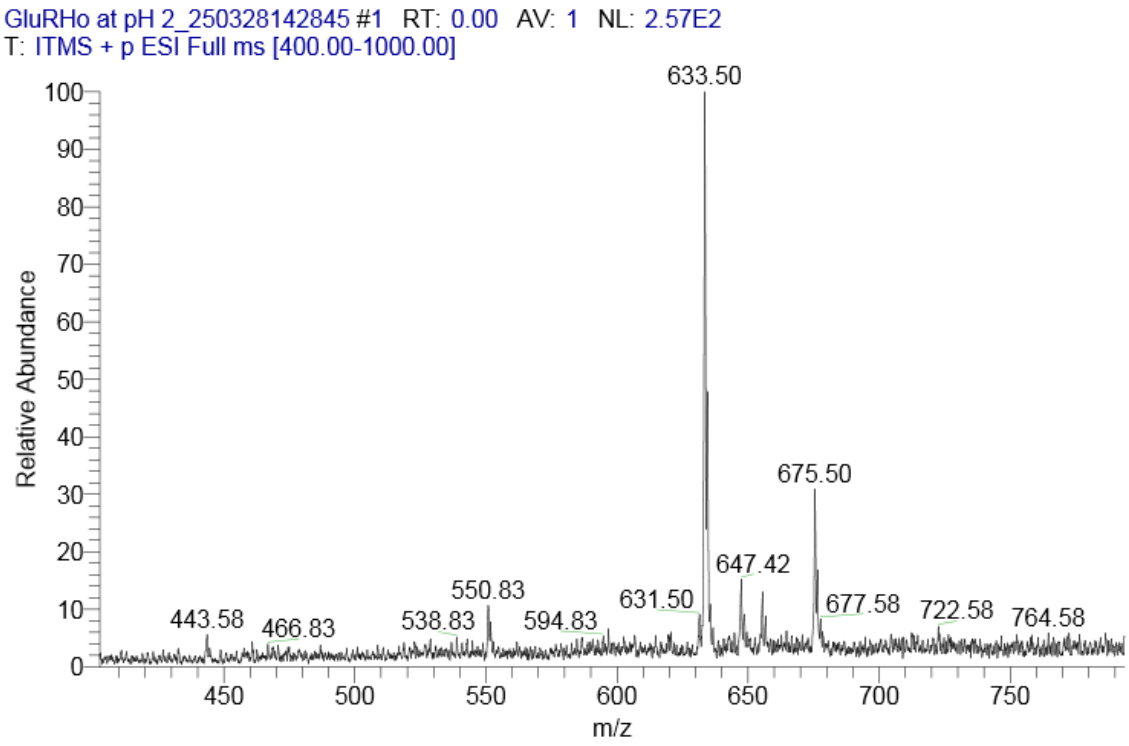

Figure S4. MS (ESI) analysis of GluRho at pH 2 in water after 15 min.

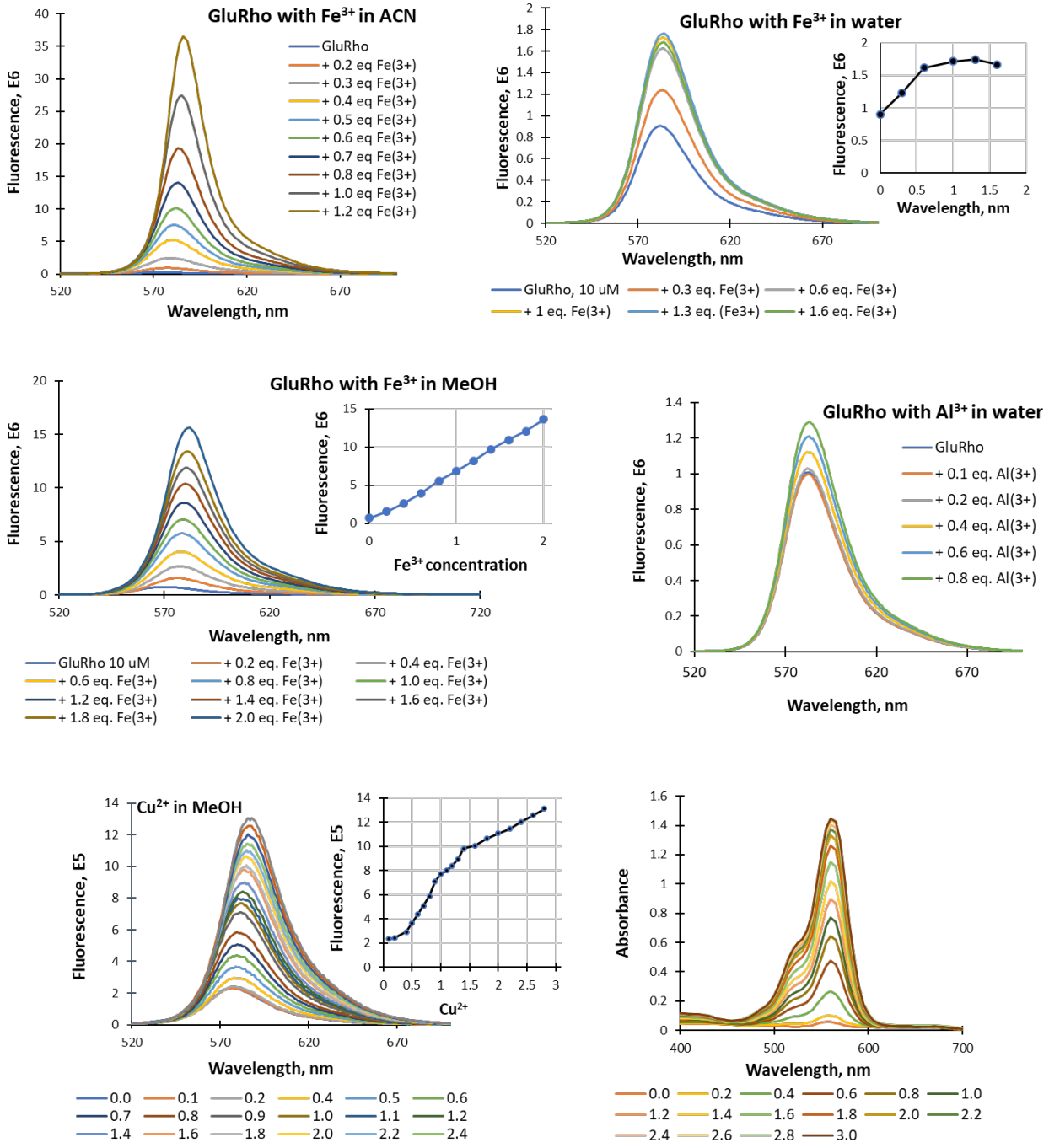

Figure S5. Impact of Fe^{3+}, Al^{3+}, and Cu^{2+}ions on GluRho (10 μM) fluorescence. GluRho was titrated with 1 μM aqueous solutions of $Fe(NO_3)_3 \cdot 9H_2O$, $Al(NO_3)_3 \cdot 9H_2O$, and $Cu(NO_3)_2$. GluRho solutions were prepared from the 10 mM stock solution in DMSO. Fluorescence recorded at 500 nm excitation.

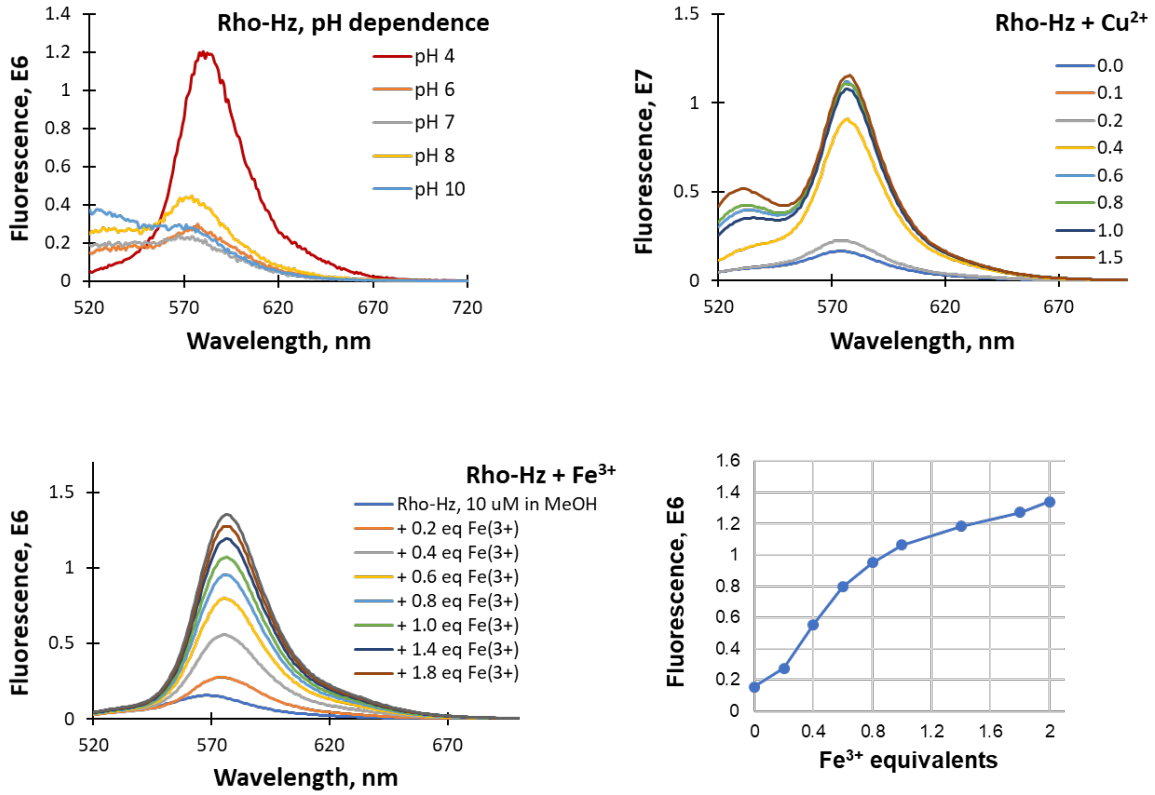

Figure S6. Rho-Hz turn-on fluorescence in the presence of ions. pH dependence established using respective aqueous buffers. For titrations using metal ions, Rho-Hz solution was prepared in MeOH. Rho-Hz (10 μM) was titrated with 1 μM aqueous solutions of $Fe(NO_3)_3 \cdot 9H_2O$ and $Cu(NO_3)_2$. Rho-Hz solutions were prepared from the 10 mM stock solution in DMSO. Fluorescence recorded at 500 nm excitation.

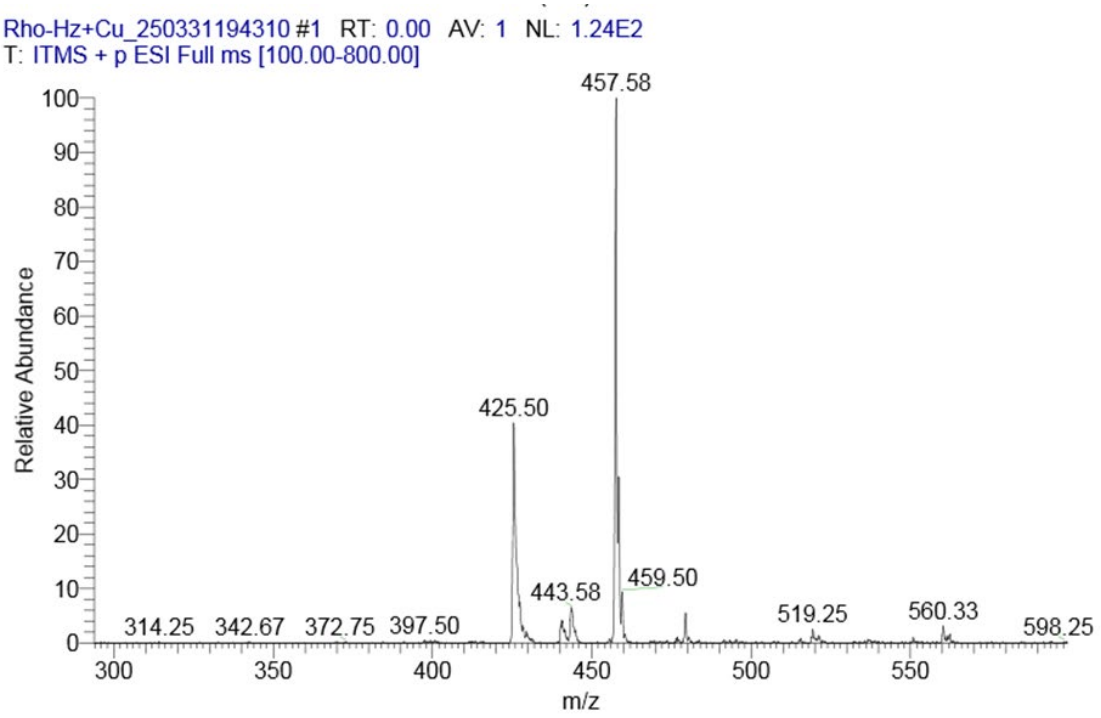

Figure S7. MS (ESI) analysis of Rho-Hz-Cu^{2+} solution in MeOH/water after 5 min. of Cu^{2+} addition.

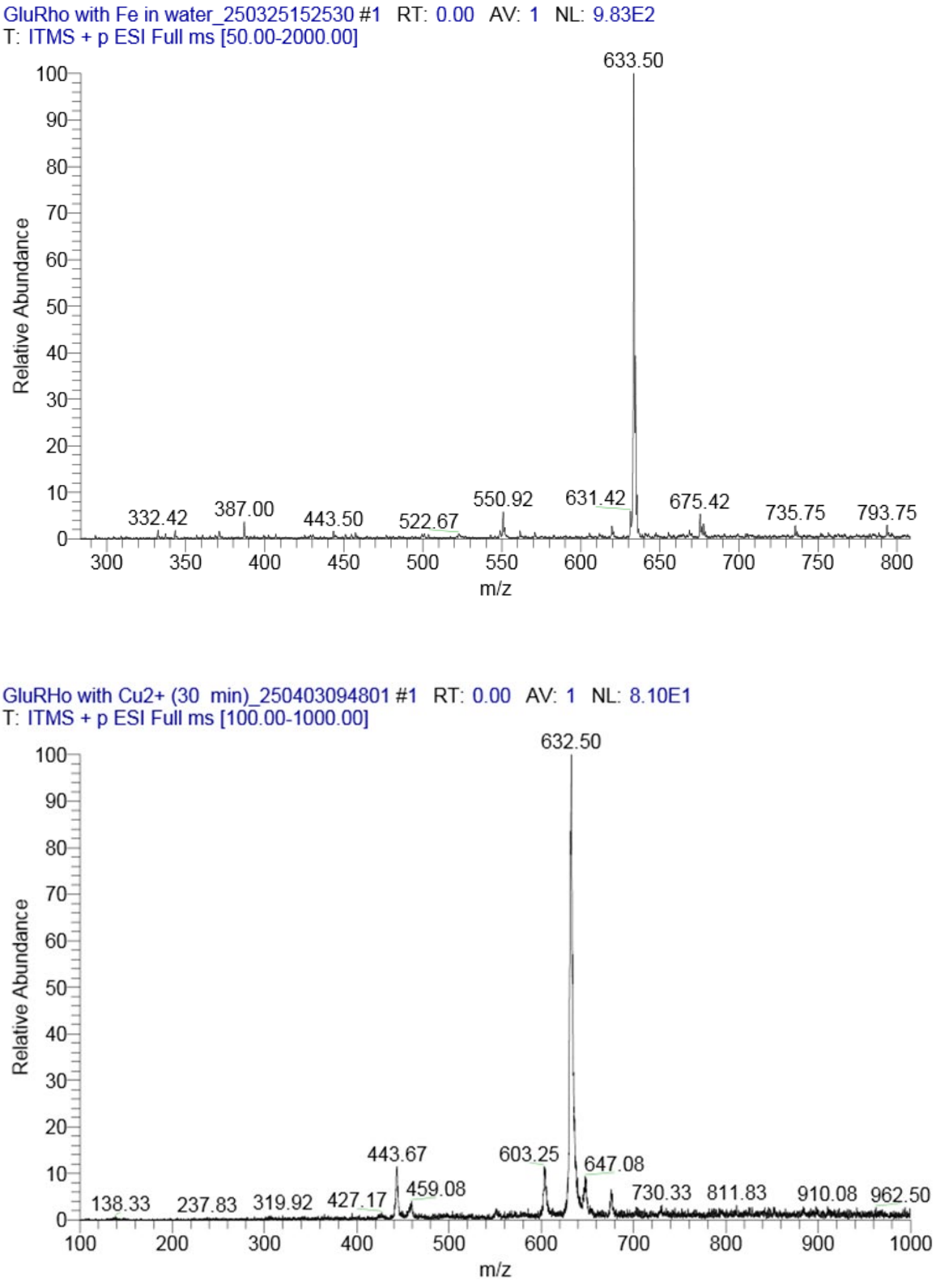

Figure S8. MS (ESI) analysis of an equimolar GluRho-Fe^{3+} and GluRho-Cu^{2+} MeOH/water solution 30 min. after metal ion addition.

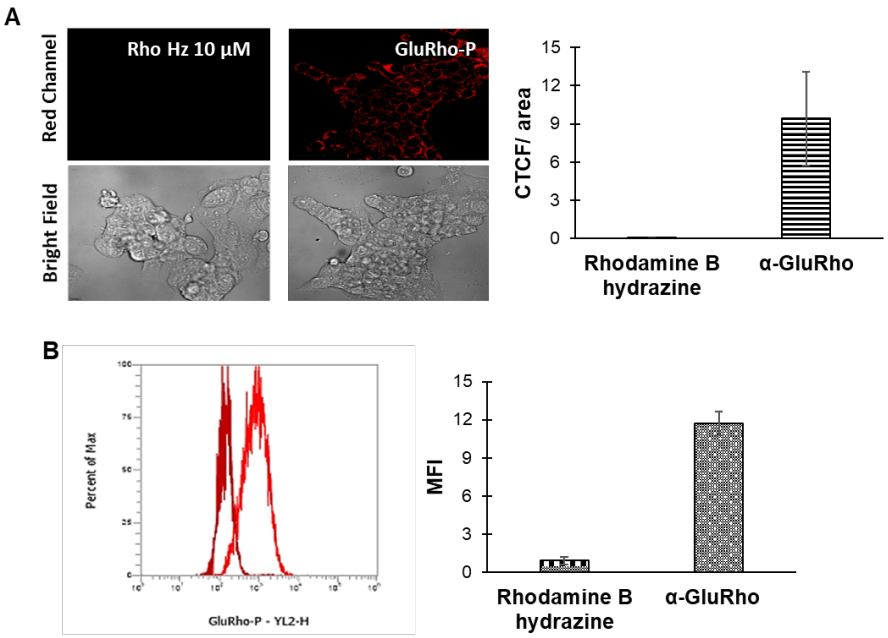

Figure S9. Analysis of sugar contribution to GluRho uptake. **A**) Uptake analysis of GluRho (2 µM) vs. Rhodamine B hydrazide (Rho-Hz, 10 µM) via confocal microscopy. Confocal images obtained using Olympus confocal microscope, 60X objective, exc. 559 nm / em. 603 nm at identical imaging parameters. The differences in probe uptake at different incubation and post-incubation times were derived through quantification of fluorescence images using ImageJ as CTCF/Area: CTCF = (Integrated Density − (Area of selected cell × Mean fluorescence of background readings))/area of the selected cell. Error bars represent the standard deviation in fluorescence between 15-20 cells. **B**) Uptake analysis via flow cytometry. Histogram represents comparative fluorescence induced by GluRho (2 µM) vs. Rho-Hz (10 µM). Fluorescence from flow cytometric analysis was quantified using YL2 filter and processed as MFI (MFI of cells treated with probe - MFI of untreated cells). Uptake evaluated in MCF7 after 15 min incubation at 37 °C. Each experiment was carried out in triplicate. Error bars represent the standard deviation between three experiments.

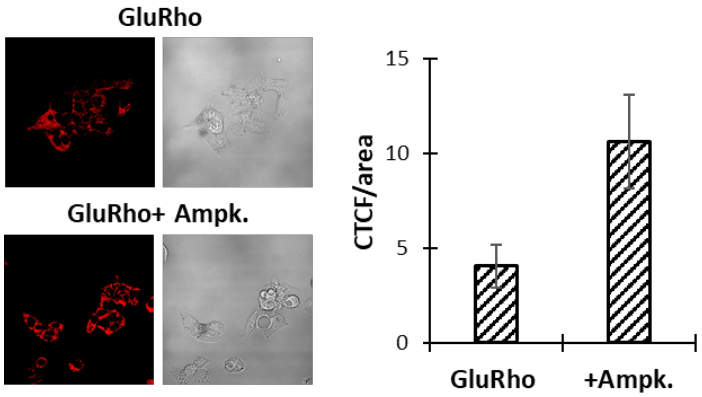

Figure S10. GluRho (2 µM) uptake in the absence and presence of ampkinone in MCF7 cells after 15 min incubation at 37 °C. Confocal images obtained using Olympus confocal microscope, 60X objective, exc. 559 nm / em. 603 nm at identical imaging parameters. The differences in probe uptake at different incubation and post-incubation times were derived through quantification of fluorescence images using ImageJ as CTCF/Area: CTCF = (Integrated Density − (Area of selected cell × Mean fluorescence of background readings))/area of the selected cell. Error bars represent the standard deviation in fluorescence between 15-20 cells.

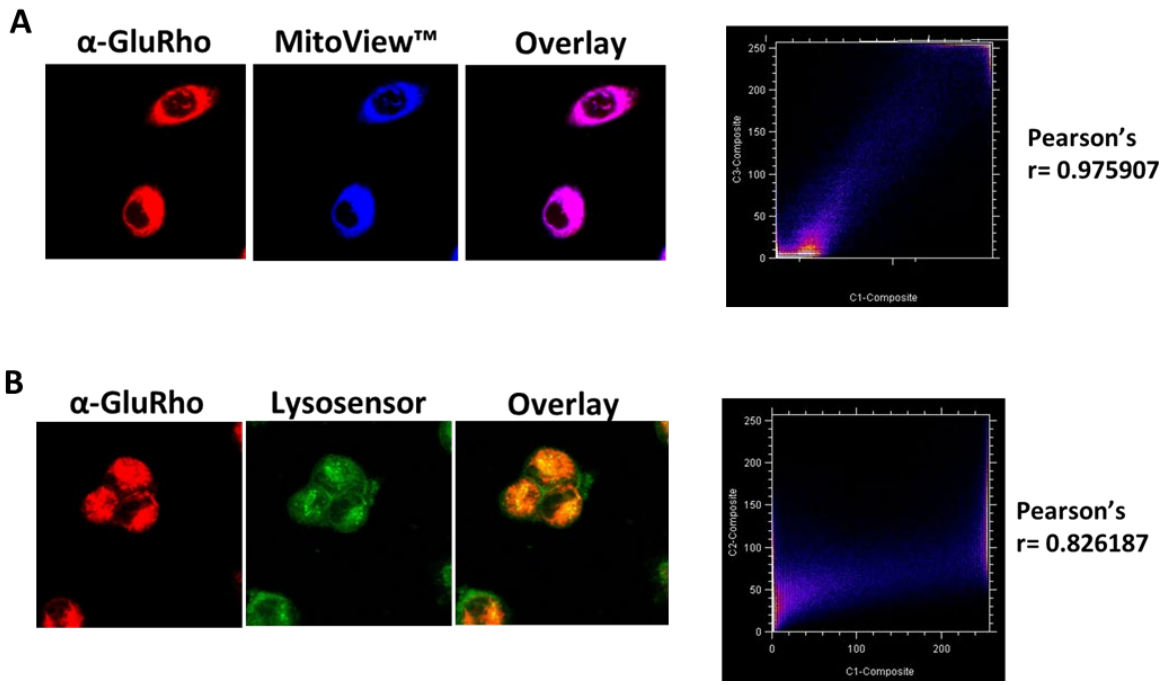

Figure S11. Localization of GluRho. **A**) Colocalization of a probe (2 μM, red) with MitoView™ (1 μM, blue). **B**) Colocalization of probes (2 μM) with Lysosensor™ Green (1 μM, green). Images were acquired using a 100X objective, exc. 559 nm /em. 603 nm for GluRho, exc. 405 nm/em. 461 nm for MitoView™, and exc. 488 nm/em. 520 nm for Lysosensor™ Green.

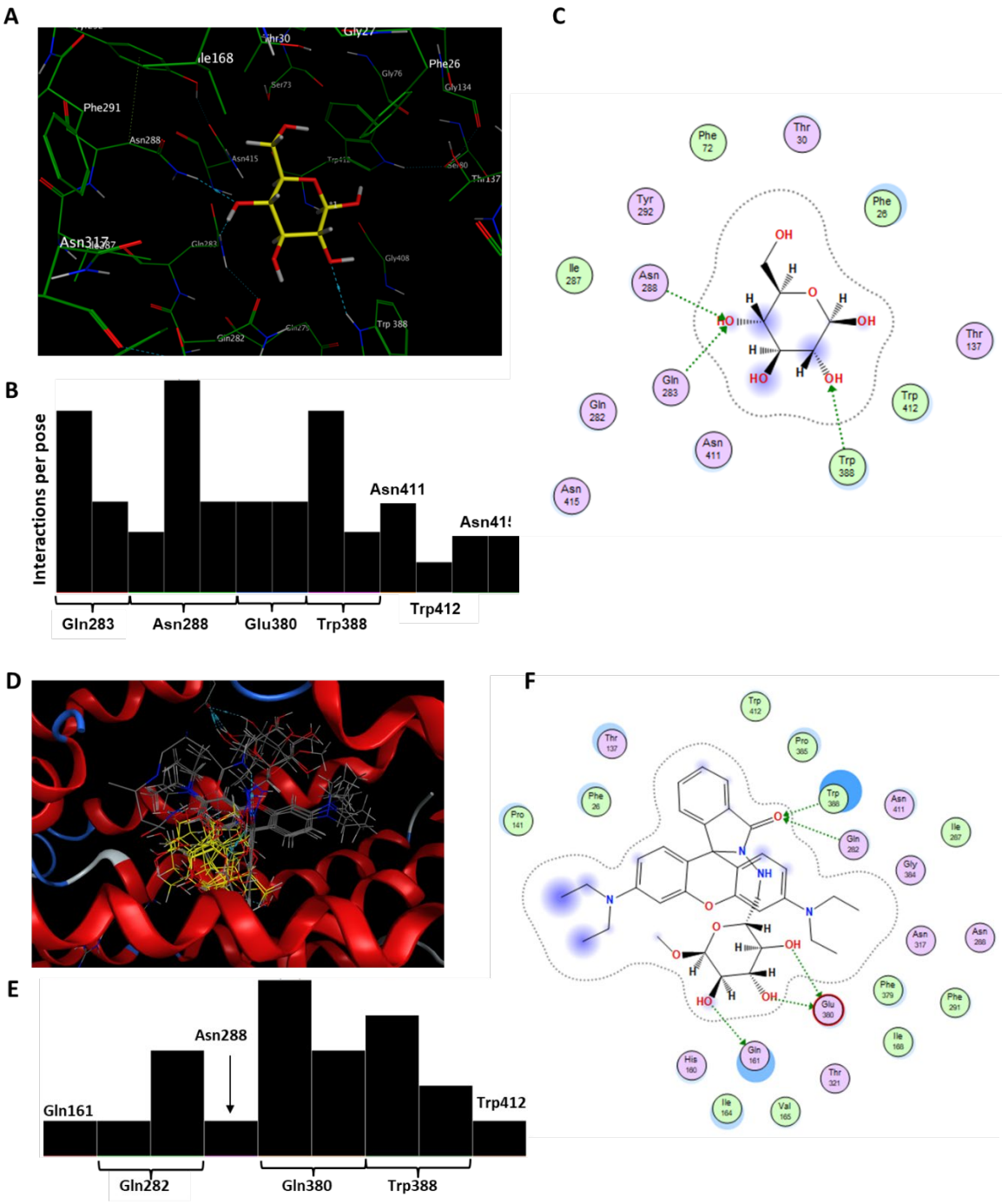

Figure S12. Docking analysis with GLUT1$_{in}$ (PDB: 4PYP)**. A**) Glucose in the substrate-binding site; **B**) Graphical summary of number of interactions per position of glucose in the docked site; **C**) Diagram projection of the major binding interactions of glucose with Glut1$_{in}$; **D**) Overlay of glucose and GluRho poses in the docked site of GLUT1$_{in}$; **E**) Graphical summary of number of interactions per position of GluRho in the docked site; **F**) Diagram projection of the major binding interactions of GluRho with Glut1$_{in}$. Docking per-formed using MOE. Imaged generated using MOE.

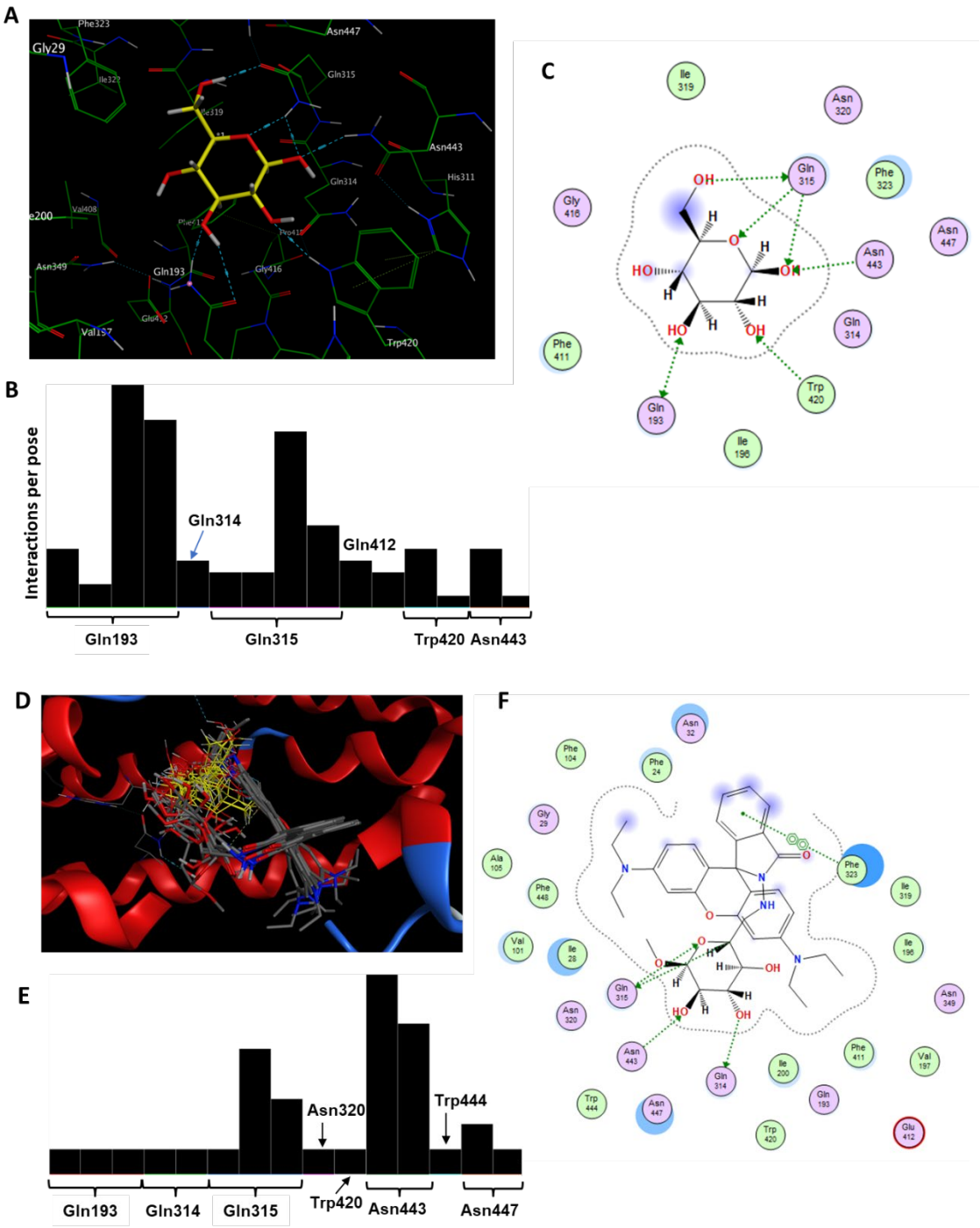

Figure S13. Docking analysis with GLUT2$_{out}$. A) Glucose in the substrate-binding site; **B**) Graphical summary of number of interactions per position of glucose in the docked site; **C**) Diagram projection of the major binding interactions of glucose with Glut2$_{out}$; **D**) Overlay of glucose and GluRho poses in the docked site of GLUT2$_{out}$; **E**) Graphical summary of number of interactions per position of GluRho in the docked site; **F**) Diagram projection of the major binding interactions of GluRho with Glut2$_{out}$. Outward-open homology model of GLUT2 was constructed based on the human outward-open GLUT3 structure (PDB: 4ZWC). Docking performed using MOE. Images, graphs, and diagrams generated using MOE.

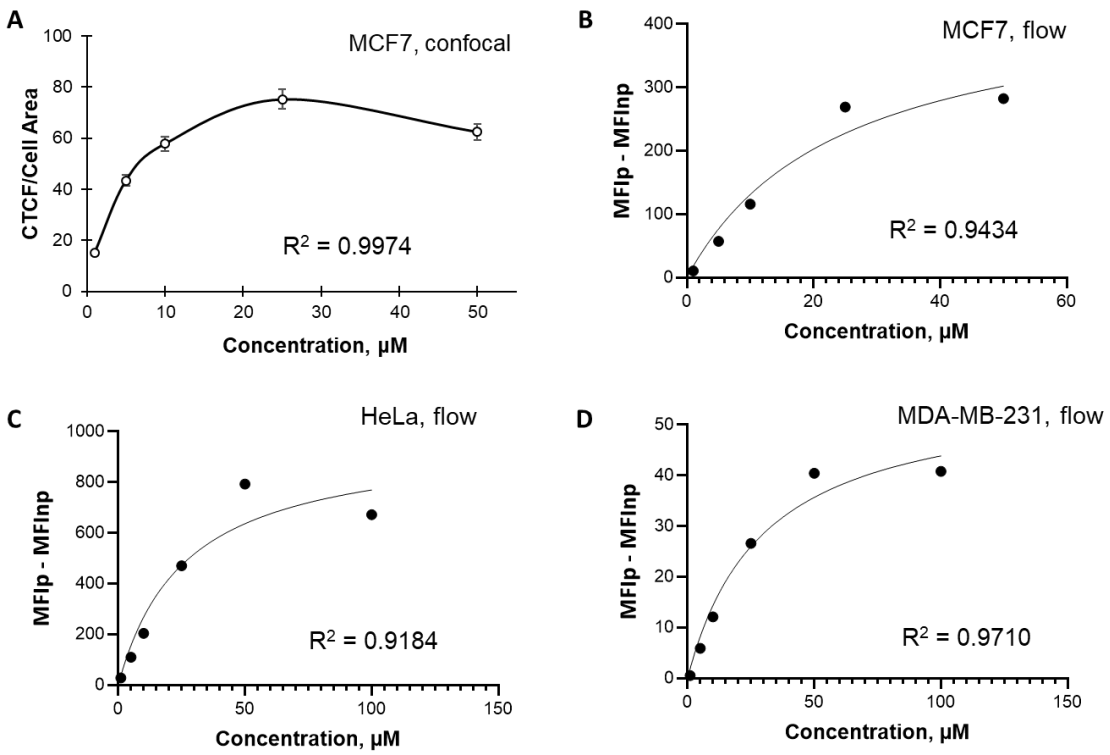

Figure S14. GluRho uptake efficiency in (A, B) MCF7 cells; (C) HeLa cells, and (D) MDA-MB-231 cells. For (A) uptake evaluated using confocal microscopy. CTCF/area represents quantified fluorescence derived from confocal images of probe-treated MCF7 cells (Figure 2D) using ImageJ. Error bars represent the standard deviation between 20 cells. For (B-D) uptake was evaluated using Attune NxT flow cytometer and is presented as dark dots. Fluorescence was quantified as MFI of cells treated with probe - MFI of untreated cells. Measurements were carried out using YL2 filter for 1000 cells per concentration. Data represents the average fluorescence for three independent repeats. Curve fitting were carried out using GraphPad Prism 10. The optimal fitting was obtained with non-linear regression analysis (Y=Bmax*X/(Kd + X)).

2. Experimental Procedures

Materials

All reagents were used as received unless otherwise stated from Sigma-Aldrich. Preparative silica chromatography was performed using SiliCycle SiliaFlash® F60 40-63 μm (230-400 mesh). Final purification of compounds was achieved with Agilent-1200 HPLC (high-pressure liquid chromatography) using a reversed-phase semi-preparative column (Phenomenex® Luna® 10 μm C18(2) 100 Å, LC Column 100 × 10 mm, Ea). ^{1}H and ^{13}C NMR spectra were recorded at room temperature with a Bruker AVANCE NEO 500 MHz spectrometer. $CDCl_3$ was used as a solvent and referenced to the corresponding residual solvent peaks (7.260 and 77.160 ppm for H- and C-NMR, respectively). The following abbreviations are used to indicate the multiplicity: s - singlet; d - doublet; t - triplet; q - quartet; m - multiplet. The coupling constants are expressed in Hertz (Hz). The high-resolution (HR) MS (ESI) spectra were obtained using a Thermo Fisher Orbitrap Elite Hybrid Ion Trap-Orbitrap Mass Spectrometer at the Chemical Advanced Resolution Methods (ChARM) Laboratory at Michigan Technological University. UV-vis spectra were recorded on a Cary 60 UV-VIS spectrophotometer from Agilent Technologies. Fluorescence spectra were obtained with a FluoroMax-4 spectrophotometer.

Breast cancer (MCF7, ATCC® HTB-22™ and MDA-MB-231, ATCC® CRM-HTB-26™) and cervical cancer (HeLa, ATCC® CCL-2™) cell lines were purchased from the American Type Cell Culture (ATCC, USA). RPMI-1640, EMEM, DMEM, Penicillin/Streptomycin, FBS (Fetal Bovine Serum), and 0.25% Trypsin-EDTA (1X) were purchased from Life Technologies, USA. Sterile DMSO (25-950-CQC, 250 mL) was purchased from Sigma-Aldrich (St Lois, MO, USA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI, USA). Glucose, glucosamine, and cytochalasin B (CyB) were purchased from Sigma-Aldrich. WZB117 and Fasentin were purchased from MolPort. G2iA and G3iA were kindly provided by Dr. Jun-yong Choe. Lysosensor™ Green DND-189 and MitoView™ dyes were purchased from Life Technologies, USA, and Biotium, USA, respectively. Poly-D-Lysine coated 35 mm confocal plates were purchased from MatTek, USA. Cell culture plates treated for increased cell attachment were purchased from VWR, USA. Confocal images were taken with Olympus FluoView FV1000 using the FluoView software. Flow cytometric analysis was carried out using Attune NxT flow cytometer.

GluRho Synthesis

*2-amino-3',6'-bis(diethylamino)spiro[isoindole-3,9'-xanthene]-1-one (**1,** Rho-Hz)*: To a stirred solution of Rhodamine B (0.48 g, 1 mmol) in EtOH (20 mL) at room temperature, excess of hydrazine hydrate (98%, 1.8 mL, 40 mmol) was added dropwise, and the mixture was heated at reflux for 18 h. Over this time, the fluorescent dark pink solution changed to transparent orange, and fluorescence disappeared, suggesting the formation of a non-fluorescent spirocyclic structure. After cooling to room temperature, the solvent was

removed under reduced pressure. The remaining residue was dissolved in $CHCl_3$ (30 mL), and the excess hydrazine hydrate was removed by washing with acid (1 M HCl). The organic phase was neutralized with 1 M NaOH (pH 8-9). The resulting precipitate of 1 was filtered, washed three times with water, and dried in the oven (~110 °C). Rhodamine B hydrazide (**1**) was obtained in 87% yield as a purple solid. HRMS (ESI): $[M+H]^+$ calc'd 456.25253; obs'd 457.25776. ^{1}H NMR (500 MHz, $CDCl_3$): δ, 7.93 - 7.94 (m, 1H), 7.44 - 7.46 (m, 2H), 7.10-7.11 (m, 1H), 6.45 -6.47 (d, 2H, J = 10), 6.42 (d, 2H, J = 2.6), 6.28 - 6.30 (dd, 2H, J_1 = 2.6, J_2 = 8.9), 3.32 - 3.36 (q, 8H, J = 7.1), 3.61 (s, 2H), 1.15 - 1.18 (t, 12H, J = 7.1). ^{13}C NMR (125 MHz, $CDCl_3$): δ, 166.14, 153.85, 151.56, 148.89, 132.50, 130.04, 128.11, 128.10, 123.83, 122.99, 108.04, 104.58, 97.98, 65.92, 44.38, 12.62.

*(2S,3S,4S,5R,6S)-3,4,5-trihydroxy-6-methoxytetrahydro-2H-pyran-2-carbaldehyde (**2**):* Sodium hydrogen carbonate (7.74 mmol) and TEMPO (6.40 µmol) were added to a stirred solution of methyl α-D-glucopyranoside (0.257 mmol) in DMF (50 mL). The reaction was cooled to 0 °C, and TCC (0.196 mmol) was added. After 7 h of continuous stirring at 0 °C, the reaction mixture was filtered and washed two times with DMF. The DMF solvent was removed under reduced pressure to obtain the desired crude aldehyde (85%). The sugar aldehyde was used for the subsequent reaction without further purification.

3',6'-bis(diethylamino)-2-((((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-methoxytetrahydro-2H-pyran-2-yl)methyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (GluRho): Aldehyde **3** (3 eq.) and Rhodamine hydrazide (1 eq.) were dissolved in MeOH (25 mL) and stirred at 0 °C for 30 min at a pH of 4 (pH adjusted using AcOH). $NaBH_3CN$ (2.4 eq) was then added in three portions (0.8 eq.) every 30 min. After the last addition, the reaction was brought to room temperature and stirred for 2 h. The solvent was removed under reduced pressure and the crude product dry-loaded onto a silica gel column and purified by column chromatography using 5% MeOH in CH_2Cl_2. Exclusively for analytical purposes, the final purification was achieved by semi-preparative HPLC to obtain the product, GluRho as a light pink solid in 20% yield. HRMS (ESI): m/z $[M + H]^+$ calc'd 632.32100; obs'd 633.32734. ^{1}H NMR (500 MHz, $CDCl_3$): δ, 7.89 - 7.90 (m, 1H), 7.44 - 7.50 (m, 2H), 7.10 - 7.11 (m, 1H), 6.40 - 6.44 (m, 4H), 6.25 - 6.28 (m, 2H, J_1 = 2.6, J_2 = 8.9), 4.58 (d, 1H, J = 3.9), 4.15 (s, 1H), 3.61 - 3.65 (t, 1H, J =9.3), 3.50 - 3.54 (t, 1H, J = 9.3), 3.43 - 3.46 (m, 1H), 3.31 - 3.35 (q, 8H, J =7.1), 3.28 (s, 3H), 2.78 - 2.91 (dABq, 2H, J_{AB} = 43, J_1 = 12.7, J_2 = 4.9), 1.14 - 1.17 (t, 12H, J = 7.1). ^{13}C NMR (125 MHz, CDCL3): δ, 166.59, 154.04, 153.88, 151.24, 149.03, 132.94, 130.29, 128.71, 128.62, 128.40, 124.04, 123.03, 108.13, 108.06, 105.29, 104.70, 99.38, 98.09, 97.80, 74.06, 72.48, 72.34, 69.25, 66.22, 55.36, 52.32, 44.47, 12.66 ppm.

Absorbance and Fluorescence Measurements

DMSO stock solution (10 mM) of GluRho was used to prepare 10 µM probe solutions. The absorption spectra of the solutions were recorded using a Cary 60 UV−Vis spectrophotometer. The fluorescence spectra (excitation at 500 nm) of these solutions were

measured using a Fluoromax-4 spectrophotometer. All the measurements were carried out at room temperature.

Analysis of GluRho and Rho-Hz activation by metal ions

Metal ion solutions were prepared from $Fe(NO_3)_3 \cdot 9H_2O$, $Al(NO_3)_3 \cdot 9H_2O$, and $Cu(NO_3)_2$ double distilled water. 10 μM GluRho or Rho-Hz solutions (10 μM, 3 ml) in water, MeOH or acetonitrile were prepared from the respective 10 mM stock solutions in DMSO. GluRho or Rho-Hz solutions (10 μM, 3 ml) were added to the quartz cuvette and titrated with an increasing ratio of metal ions. The change in GluRho fluorescence in response to the increased concentration of metal ions was recorded immediately after addition.

Cell Culture

All cultures were supplemented with 10% fetal bovine serum (FBS), 10,000 IU/mL penicillin, and 10,000 μg/mL streptomycin to prevent bacterial contamination. The MCF7 and MDA-MB231 cell lines were cultured using RPMI-1640. HeLa cells were grown and maintained using a DMEM medium. Cells were collected at ∼80% confluence of the 10 mm tissue culture plate using 0.25% trypsin−EDTA (2 mL). The trypsin fraction was diluted with fresh culture medium to 5 mL, and cells were pelleted by centrifugation (1600 rpm, 5 min). After centrifugation, the cells were reconstituted in fresh culture medium (5 mL) and seeded at the desired density for confocal imaging and flow cytometric studies. The cells were then incubated for 48 h at 37 °C, 65% relative humidity, and under 5% CO_2 in the ATCC-suggested respective culture media according to the ATCC protocol.

Confocal Imaging/Uptake Analysis

Cells were collected from culture dishes, pelleted by centrifugation, reconstituted in fresh culture medium (5 mL), and seeded (150,000 cells) per 35 mm glass-bottom confocal dish (MatTek). Cells were incubated at 37 °C in 5% CO_2 After 48 h, the cell medium was removed, and the probe solution (1 mL) was added. Cells were incubated with probes at 37 °C for a specific time interval. For all studies, the cells were imaged directly after incubation without removing probe solution and subsequent cell wash. Images were taken with Olympus FluoView FV1000 using the FluoView software. A 60× oil-suspended lens was used to acquire fluorescent images with the following conditions: ex. 559 nm, em. 568 nm, 20 μs/pixel, C.A. = 120 μm. The obtained fluorescence images were quantified using ImageJ. Fluorescence was calculated as CTCF/area where CFCT = corrected total cell fluorescence (CTCF = integrated density − (area of selected cell × mean fluorescence of background readings)). This procedure was done for cells by selecting regions of interest in the single image. Quantified data represent an average fluorescence of 7−15 cells per image. At least two images per sample were processed. All images were taken at the same imaging settings.

Colocalization Analysis

Intracellular localization of GluRho probe was performed using MitoView™ and Lysosensor™ Green DND 189 in MCF7, MDA-MB-231, and HeLa cells. For the GluRho and MitoView co-stain, a solution of GluRho (2 μM) and MitoView (1 μM) in cell culture media was added to cells and incubated at 37 °C for 15 min. For the GluRho and Lysosensor co-stain, a solution of GluRho (2 μM) and Lysosensor Green (1 μM) in cell media was added to cells and incubated at 37 °C for 15 min. After incubation, the cells were imaged immediately without probe removal. Pearson's correlation coefficient was calculated using the Colocalization Finder ImageJ plugin.

Temperature studies

GluRho solution (2 μM) was prepared in complete cell culture media (1 mL) using 5 mM probe stock solutions in DMSO. MCF7 cells were seeded in confocal plates. Before treatment with probes, cells were incubated in the refrigerator at ~4 °C for 30 min. After that, the culture medium was discarded, and a cooled solution of a probe in the complete culture medium was added. Cells were incubated in the refrigerator for 15 min. After treatment, the cells were imaged immediately.

Uptake Analysis in the Presence of Additives (Competitive substrates, inhibitors or inducers)

All compound stock solutions are prepared in DMSO: 5 mM for CytB, WZB 117, G2iA and G3iA; 10 mM for fasentin and ampkinone. For treatment, each additive compound solution was used to prepare an additive/probe mixture in complete cell culture media (1 mL) that was added to MCF7 cells seeded in glass-bottom confocal dishes after removal of the cell culture medium. Cells were incubated at 37 °C for 15 min. After incubation, the treated cells were immediately taken for fluorescence imaging. Cells treated with a 0.5 μM GluRho probe were used as a control.

Time-dependent fluorescence analysis

GluRho solution (2 μM) was prepared in complete cell culture media (1 mL) using 10 mM probe stock solutions in DMSO. For all probe solutions, DMSO concentration was kept below 1%. For MCF7 treatment, cells seeded in glass-bottom confocal dishes were used. Cell media was removed, and a probe solution (1 mL) was added. Cells were incubated with probe solution at various time points (10, 20, 30, 40, 50 and 60 min) at 37 °C. After incubation, the cells were imaged immediately without probe removal.

Uptake Kinetics Analysis

GluRho solutions (1, 5, 10, 25, and 50 μM) were prepared in complete cell culture media (1 mL) using 5 mM probe stock solutions in DMSO. For treatment, MCF7 cells seeded in glass-bottom confocal dishes were used. Cell media was removed, and a probe solution (1

mL) was added. Cells were incubated with probe solution at 37°C for 15 min. After incubation, the cells were imaged immediately without probe removal.

Drosophila melanogaster larvae uptake studies

First-instar larvae of the *Drosophila melanogaster* wild-type stock Canton-S were collected after hatching from the eggs, washed 2× in PBS solution, and incubated in 400 µL of a PBS-based probe solution (with and without sugar solutions and ampkinone added) for 2 h. After the incubation, the larvae were washed 3× in PBS, mounted alive on slides, and covered with a coverslip. Fluorescence imaging was performed with a confocal fluorescence microscope (Olympus) using a 10× objective lens, Alexa Fluor 568 filter, and 24% laser (559 nm) intensity (C.A. = 219 µm). Images recorded at λ_{ex}/ λ_{em} 559/603 nm. All images were taken at the same exposure time.

Modeling

The modeling was carried out using the MOE platform; energy-refined structures for glucose and GluRho were generated using the conformational search function, and then the docking poses were generated and refined using the following parameters. The initial ligand placement within the binding site was carried out using the Triangle Matcher algorithm. The poses were scored using the London dG scoring function, which estimates binding free energy (ΔG) based on hydrophobic interactions, hydrogen bonding, and ligand flexibility penalties. The ligand poses were refined using the Rigid Receptor method, and the refined poses were scored with the GB/WSA dG scoring function, which calculates binding free energy by incorporating solvation effects via the Generalized Born model and hydrophobic surface area contributions.

3. Copies of ^{1}H and ^{13}C NMR spectra

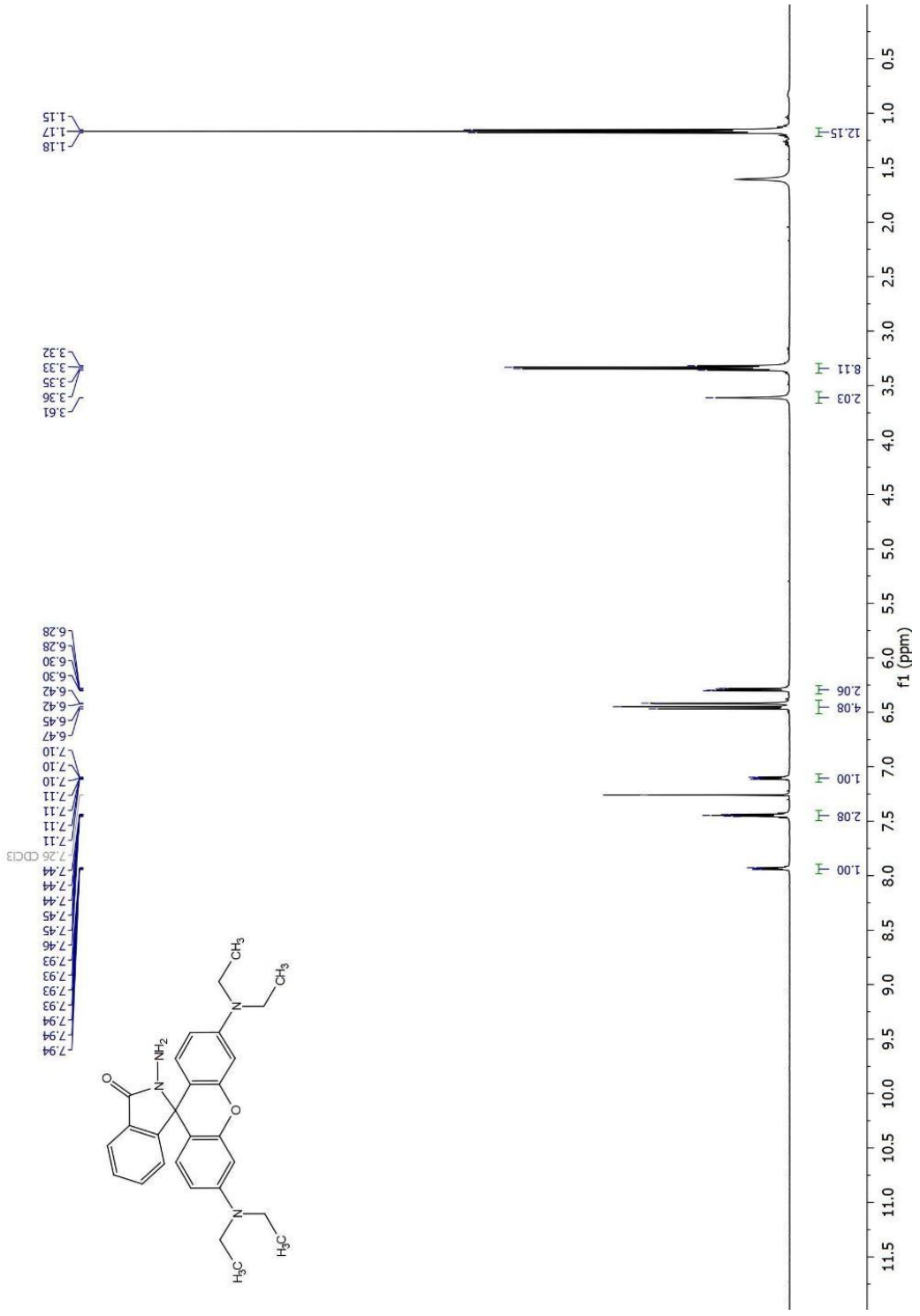

Figure S15. ^{1}H NMR spectrum of Rhodamine B Hydrazine (Rho-Hz), $CDCl_3$, 500 MHz

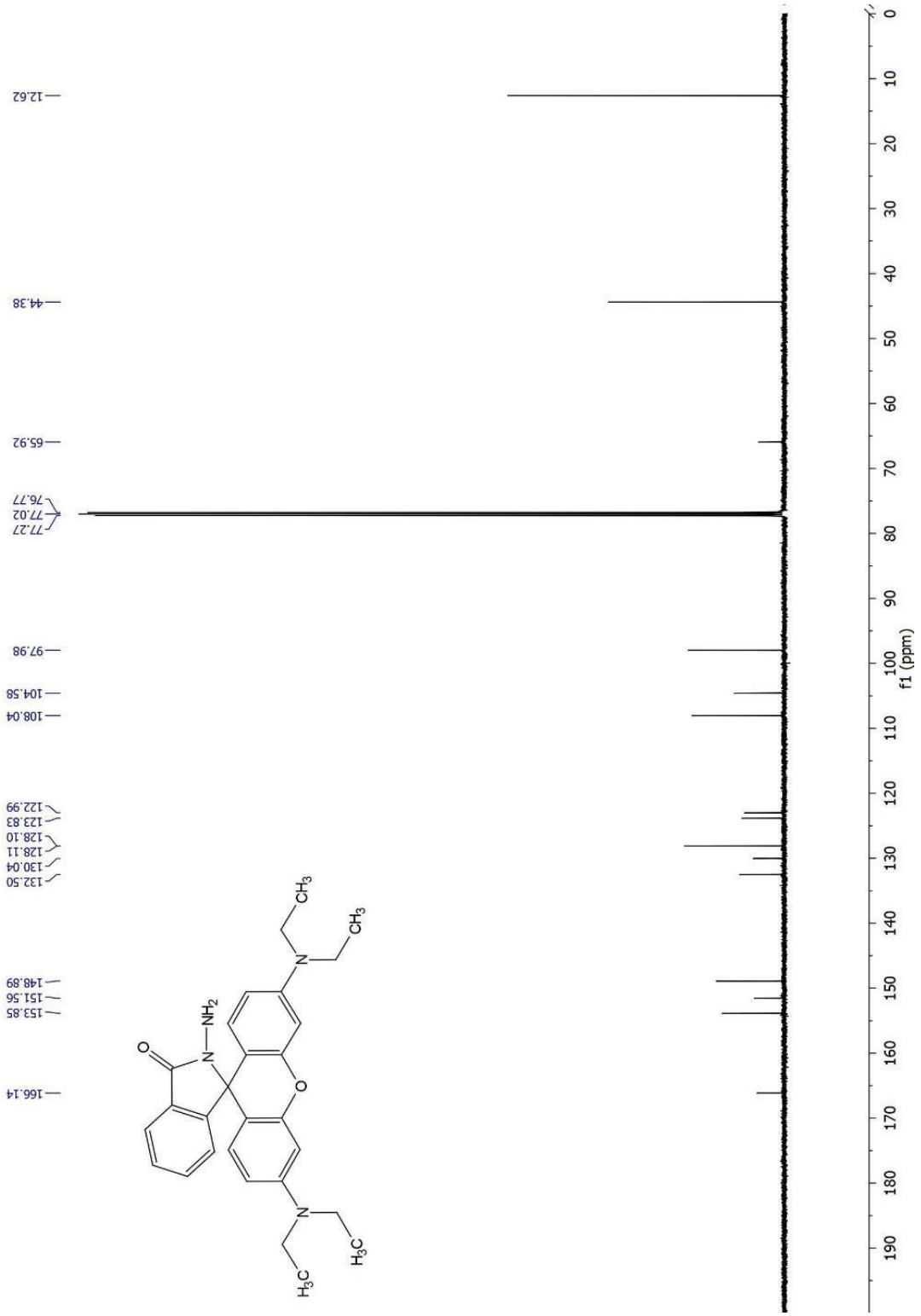

Figure S16. ^{13}C NMR spectrum of Rhodamine B Hydrazine (Rho-Hz), $CDCl_3$, 125 MHz

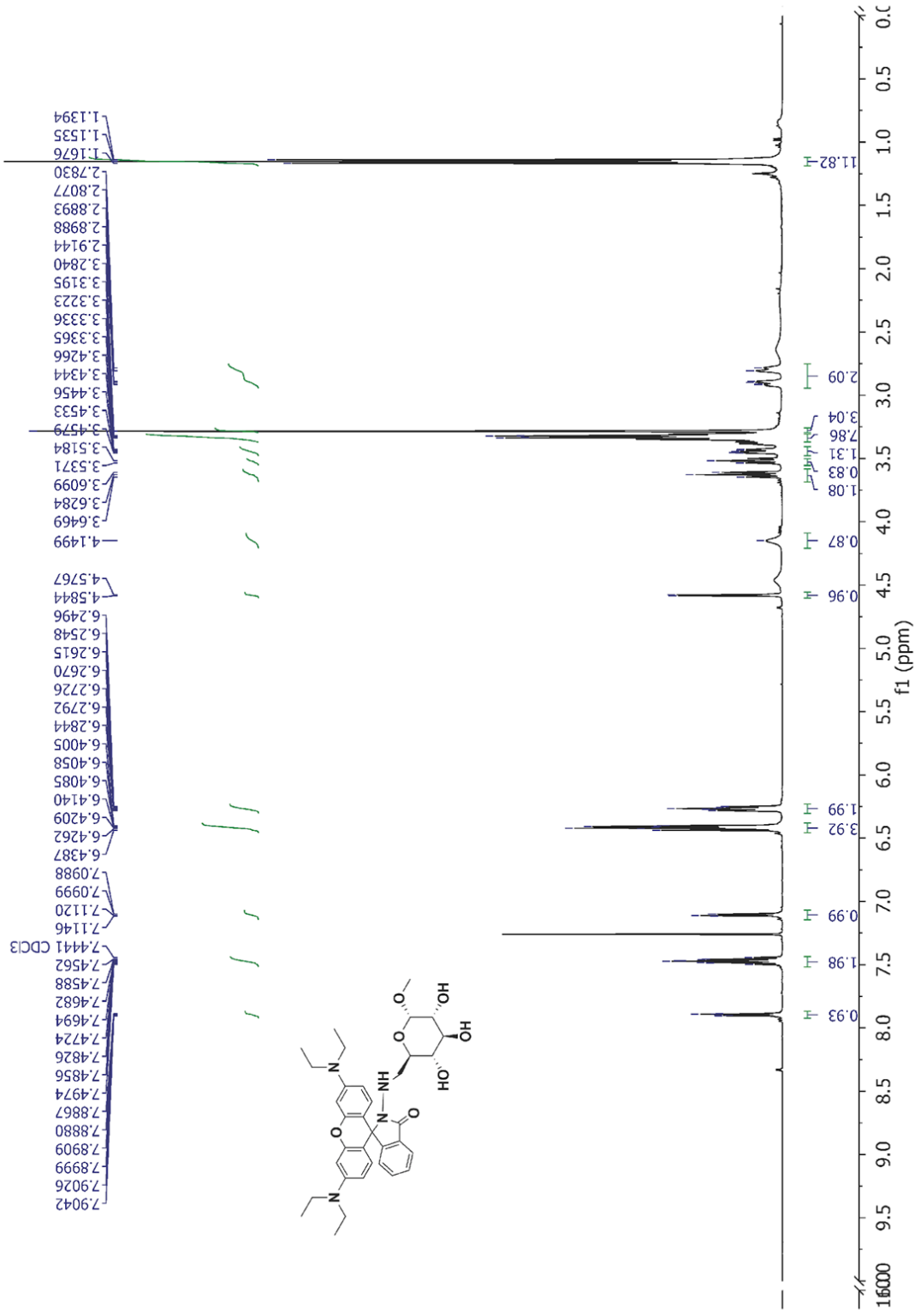

Figure S17. ^{1}H NMR spectrum of GluRho, $CDCl_3$, 500 MHz

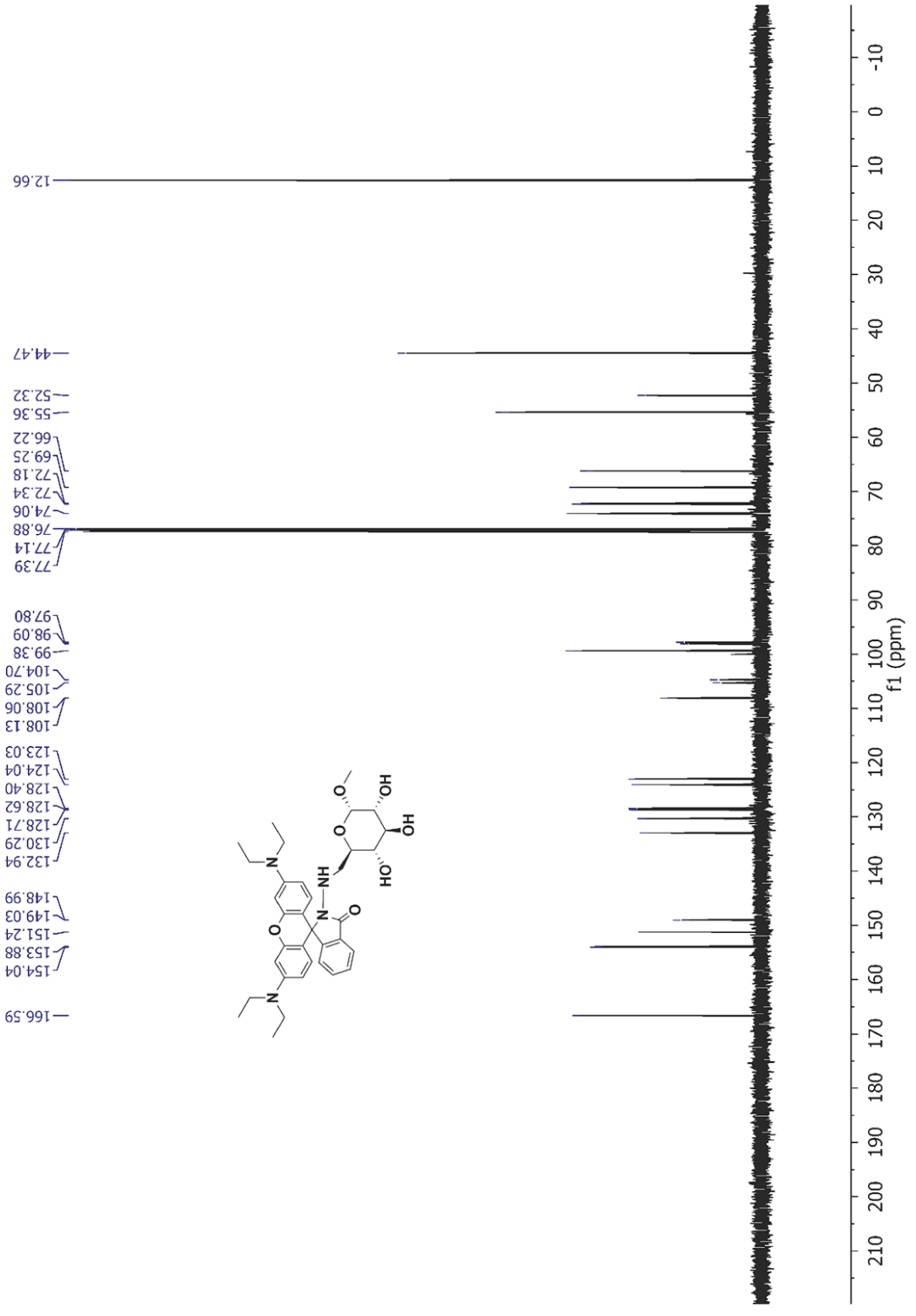

Figure S18. ^{13}C NMR spectrum of GluRho, $CDCl_3$, 125 MHz