Supporting Information

A New Highly Selective Fluorescent K^+ Sensor

Xianfeng Zhou, Fengyu Su, Yanqing Tian*, Cody Youngbull, Roger H. Johnson, Deirdre R. Meldrum

Center for Biosignatures Discovery Automation, Biodesign Institute,
Arizona State University, Tempe, AZ 85287

*To whom all correspondence should be addressed. Phone: 480-965-9601. Fax: 480-727-6588. E-mail: Yanqing.tian@asu.edu

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S-Figure 10. Time-dependent fluorescence of U87MG cells stimulated by nigericin observed under confocal fluorescence microscope. a is time of 0 before the addition of nigericin; b, c, d, e, f, and g are time of 1, 3, 5, 10, 15, and 25 minutes, respectively, after the addition of nigericin (20 μM, final concentration) into the 20 mM KCl-containing medium; h gives the average fluorescence intensity ratios measured by ImageJ. I_0 is the average fluorescence intensity from figure a; I is the average fluorescence intensity at different times. **P16**

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1. Experimental Section:

1.1 Reagents. TAC-CHO and 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) were synthesized according to known procedures.[1,2] Lysosensor SM3 was prepared according to our published procedure.[3] KCl, NaCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, $ZnCl_2$, $CuCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nigericin, bumetanide, and ouabain were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342, PBFI, and MitroTracker® Green FM were ordered from Invitrogen (Carlsbad, CA).

1.2 Instruments. A Varian liquid-state NMR operated at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR was used for NMR spectra measurements. High resolution mass spectrometry (HRMS) was performed by the ASU Mass Spectrometry Laboratory. A Shimadzu UV-3600 UV-Vis-NIR spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) was used for absorption spectra measurements. A Shimadzu RF-5301 spetrofluorophotometer was used for fluorescence measurements.

1.3 Synthesis of KS2. In a round-bottom flask fitted with a reflux condenser, TAC-CHO (100 mg, 138.5 μmol), and TCF (33.12 mg, 166.2 μmol) were dissolved in 2 mL of absolute ethanol. NaOH (2 mg) was added to this mixture and refluxed for 12 hrs. The deep violet solution was extracted with

dichloromethane from water, and the organic layer was dried with $MgSO_4$. The crude product was purified by column chromatography over silica gel to get a dark-purple solid (62 mg, yield: 49.6%); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.58 (d, 1H), 6.6-7.2 (m, 10H), 3.0-4.2 (m, 39H), 2.23 (s, 6H), 1.74 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 175.86, 173.91, 152.83, 149.40, 147.52, 144.26, 138.29, 132.74, 126.39, 124.96, 121.70, 121.45, 117.24, 114.27, 112.70, 112.35, 111.60, 111.13, 110.35, 71.20, 70.80, 69.99, 68.25, 67.10, 58.99, 55.50, 53.66, 53.46, 29.68, 26.70, 21.03. HRMS (APCI+): m/e calculated for $C_{51}H_{62}N_6O_9$ (M+H): 902.4578; found. 925.4497 (M + Na).

1.4 Quantum efficieny determination. The fluorescence quantum yields (η) of samples in solutions were recorded by using Rhodamine B in ethanol ($\eta = 0.65$) [4] excited at 540 nm and were calculated according to the following equation [5]:

$$\eta_s = \eta_r \left(\frac{A_r}{A_s} \right) \left(\frac{I_s}{I_r} \right) \left(\frac{n_s^2}{n_r^2} \right)$$

where (η_r) and (η_s) are the fluorescence quantum yields of standards and the samples, respectively. A_r and A_s are the absorbance of the standards and the measured samples at the excitation wavelength, respectively. I_r and I_s are the integrated emission intensities of standards and the samples, respectively. n_r and n_s are the refractive indices of the corresponding solvents present in the solutions, respectively. The experimental error was approximately 10%.

1.5 Sensor responses: 50 μL of KS2 dissolved in DMSO resulting in a concentration of 200 μM which was then added into 2 mL of HEPES (10 mM, pH 7.2) buffer. This resulted in the final KS2 concentration to be 5 μM in the buffer. Different metal ion concentrations were added into the solution for studying KS2's responses. Although the reaction of KS2 and the metal ion was rapid (usually within seconds), the spectra was measured after 2 minutes to ensure stability. KS2/metal ion complexes were excited at 561 nm and emissions were collected from 600 to 750 nm.

1.6 Cell culture for imaging. U87MG cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 100 u/mL penicillin, 2 mM L-glutamine (Sigma-Aldrich), and incubated at 37 °C in 5% CO_2 atmosphere. Cells were then seeded onto 96 well plates at 10,000 cells per well, and incubated for 1 day at 37 °C. Sensors dissolved in DMSO were added to the medium to make the sensor concentrations in a range of 0.5 - 5 μM. 10 min of internalization was found to be sufficient for achieving satisfactory images. To achieve images with satisfactory signal-to-noise ratio, a sensor concentration of 4 μM was usually used for intracellular study.

A study was conducted to test whether or not the sensor had specific subcellular distributions in cells: cells were co-stained using the sensor in combination with Hoechst 33342, Lysosensor SM3, and MitoTracker® Green FM, respectively. To co-stain the nuclei, Hoechst 33342 was used. Cells were4

first internalized with KS2 (4 μM in cell culture medium) for 10 minutes. After removing medium and washing the cells with fresh medium, Hoechst 33342 dissolved in the fresh medium and was then added into the wells to stain cell nuclei for 30 min at 37°C. In order to co-stain the cell's mitochondria, MitoTracker® Green FM was used. The cells were treated in the same manner as the Hoechst staining method, but with a MitoTracker® Green FM in DMSO solution. To co-stain the lysosomes, Lysosensor SM3 was used. Again the cells were treated using the previously stated protocol while using a SM3 in DMSO solution. The resulting concentrations of Hoechst 33342, MitoTracker® Green FM, and Lysosensor SM3 in the cell medium were 10 μM, 50 nM, and 2 μM respectively.

Under a Nikon Eclipse TE2000E confocal fluorescence microscope (Melville, NY), Hoechst 33342 was excited at 405 nm and its blue emission was collected using a 450/35 nm filter set. Sensor (KS2) was excited at 561 nm and its red emission was collected using a 605/75 nm filter set. Lysosensor SM3 was excited at 405 nm and its green emission was collected using a 515/30 nm filter set. And MitoTracker® Green FM was excited at 488 nm and its green emission was collected using a 515/30 nm filter set.

1.7 Possible interference of PBFI on KS2 uptake: For this comparison, two independent experiments were carried out. In the first experiment, cells were internalized with KS2 for 10 minutes at a concentration of 2 μM at 37 °C for 10 minutes. Then the medium containing the sensor was removed, and new medium was added for imaging at 37 °C. While in the second experiment, the cells were first internalized with PBFI at a concentration of 10 μM in cell culture medium for 30 minutes at 37°C. Then KS2 (at a concentration of 2 μM) was added to the cell medium. Cells were further incubated at 37 °C for 10 minutes. After which the sensor and medium were removed, and new medium was added for imaging at 37 °C. The excitation wavelength was 561 nm and the emissions were collected using a 605/75 nm filter set. Both of the experiments were carried out using the same optical procedure.

1.8 Monitoring the intracellular K^+ efflux using KS2. U87MG cells were seeded onto 96 well plates at 10,000 cells per well in 100 μL medium overnight at 37°C. On the following day, the cells were internalized with KS2 (4 μM) for 10 min at 37°C. A mixture of nigericin, bumetanide, and ouabain was then added. Final concentrations of nigericin, bumetanide, and ouabain in the cell culture medium were 5 μM, 10 μM, and 10 μM, respectively. Fluorescence in cells was visualized by Nikon Eclipse TE2000E confocal fluorescence microscope (Melville, NY) at 37 °C.

1.9. Monitoring the intracellular K^+ influx using KS2. U87MG cells were seeded onto 96 well plates at 10,000 cells per well in 100 μL medium overnight at 37°C. On the following day, the cells were internalized with KS2 (4 μM) for 10 min at 37°C. The influx stimulator (isoproterenol in KCl-containing PBS buffer) was added. Final concentrations of the added isoproterenol and KCl in the cell

culture medium were 5 μM and 20 mM, respectively. Fluorescence in cells was visualized by Nikon Eclipse TE2000E confocal fluorescence microscope (Melville, NY) at 37 °C.

1.10 Viable cell counts using Trypan Blue. To cell culture medium (100 μL) with eukaryotic cells in a 96 well microplate with 10,000 cells/well that had internalized the sensor for 2 hours, 10 mL of 0.4% Trypan Blue stain was added and mixed thoroughly with the medium. After standing for 5 min at room temperature (23 °C), the cells were imaged using an optical microscope in bright field mode. Dead cells appeared blue from the Trypan Blue stain, whereas healthy cells appeared transparent as a result of the cell's resistance to stain. The cells were then counted and the ratio of dead cells to live cells was calculated. Each experiment was repeated three times.

2. Discussion

2.1 About KS2's subcellular distribution.

There are some co-localizations of KS2 with mitochondria and lysosome, since the mitochondria and lysosome also contain potassium ions. These results indicated the non-specific cellular distribution of the potassium ion sensor, KS2. The non-specific distribution of KS2 indicated that it can be used to monitor potassium ions in the cytoplasm and in any of these compartments.

2.2 Possible further material development direction.

The current KS2 exhibited low quantum efficiency. Further improvement of the quantum yields while keeping a K_d value around 100 mM is necessary although quite challenging.

3. References:

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2. Gopalan, P.; Katz, H. E.; McGee, D. J.; Erben, C.; Zielinski, T.; Bousquet, D.; Muller, D.; Grazul, J.; Olsson, Y. *J. Am. Chem. Soc.* **2004**, *126*, 1741-1747.
3. Tian, Y.; Su, F.; Weber, W.; Nandakumar, V.; Shumway, B. R.; Jin, Y.; Zhou, X.; Holl, M. R.; Johnson, R. H.; Meldrum, D. R. *Biomaterials.* **2010**, *31*, 7411-7422.
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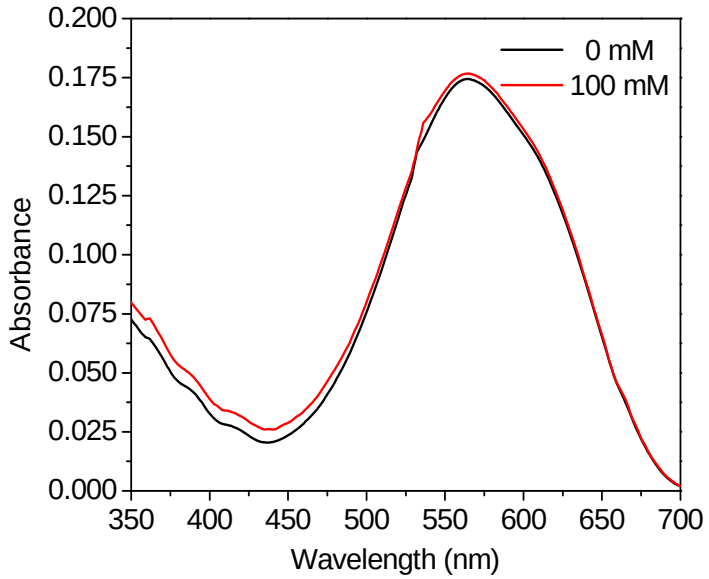
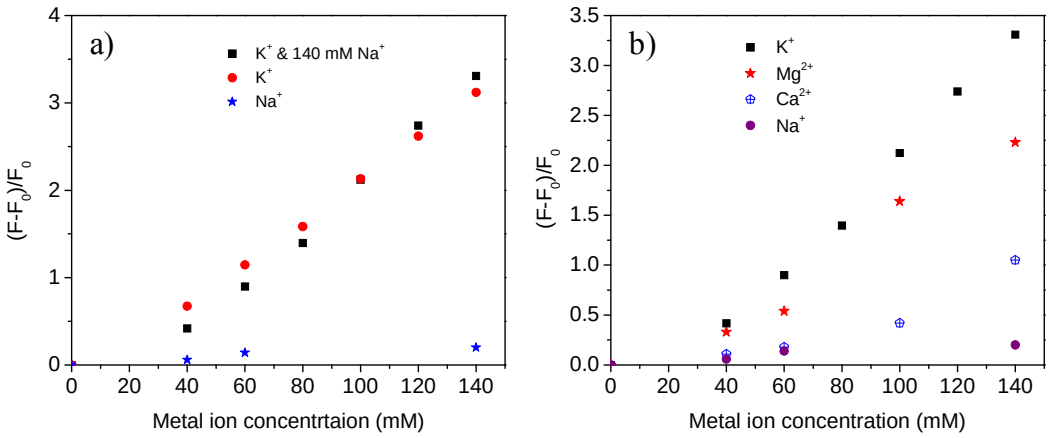
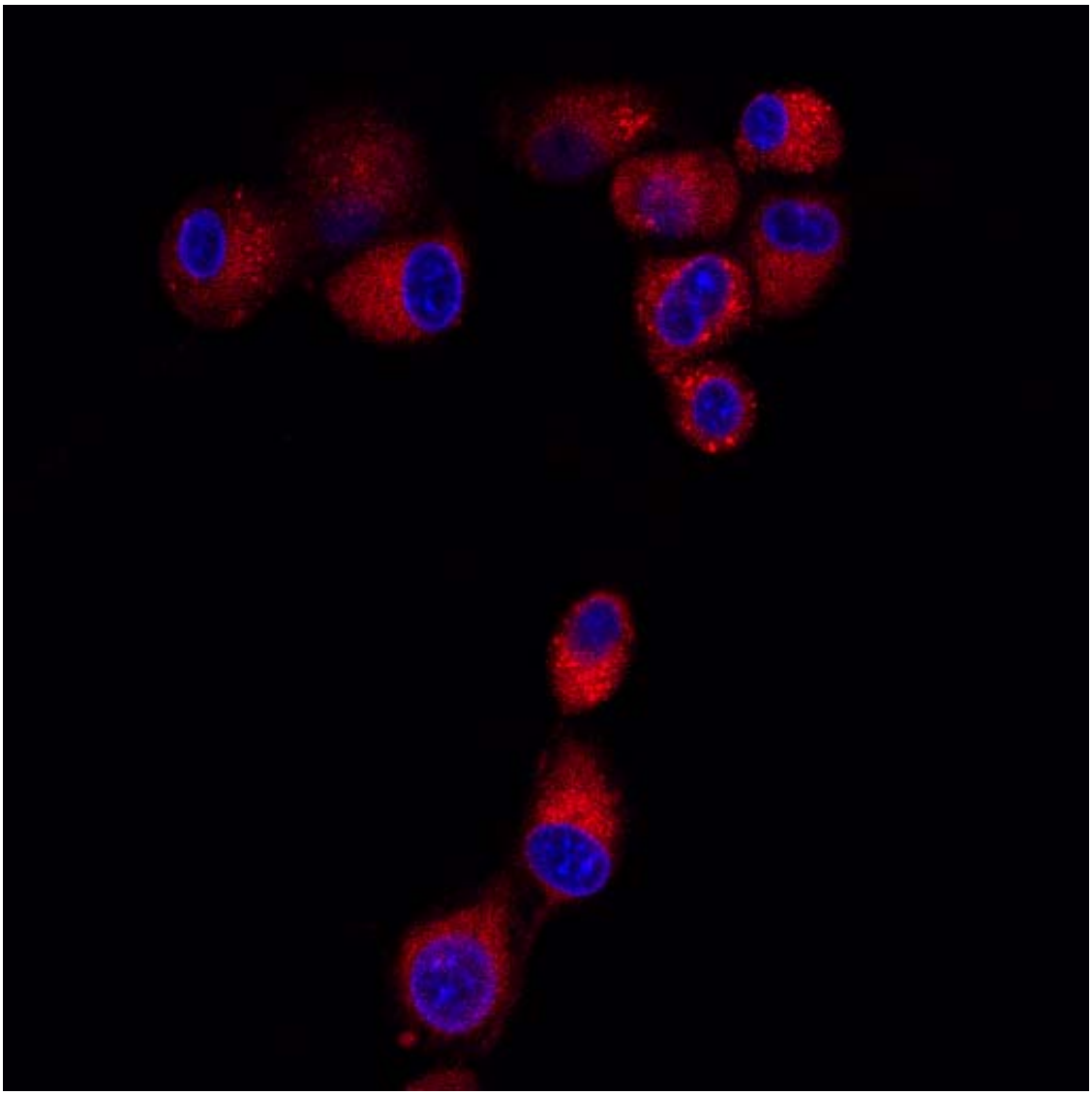
5. Joshi, H. S.; Jamshidi, R.; Tor, Y. *Angew. Chem. Int. Ed*. **1999**, *38*, 2721-2725.



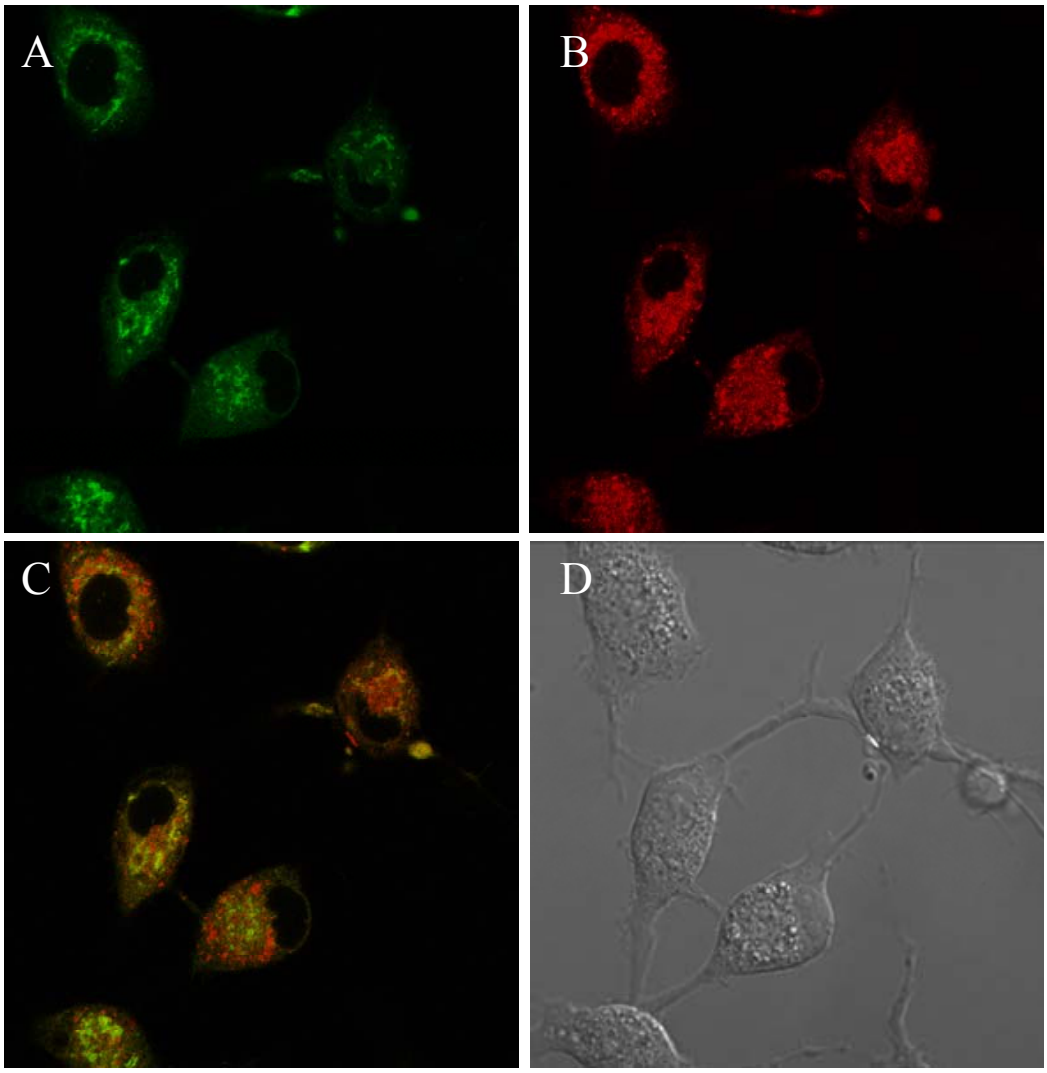
S-Figure 1. UV-Vis spectra of KS2 (5 μM) before and after the interaction with 100 mM KCl.



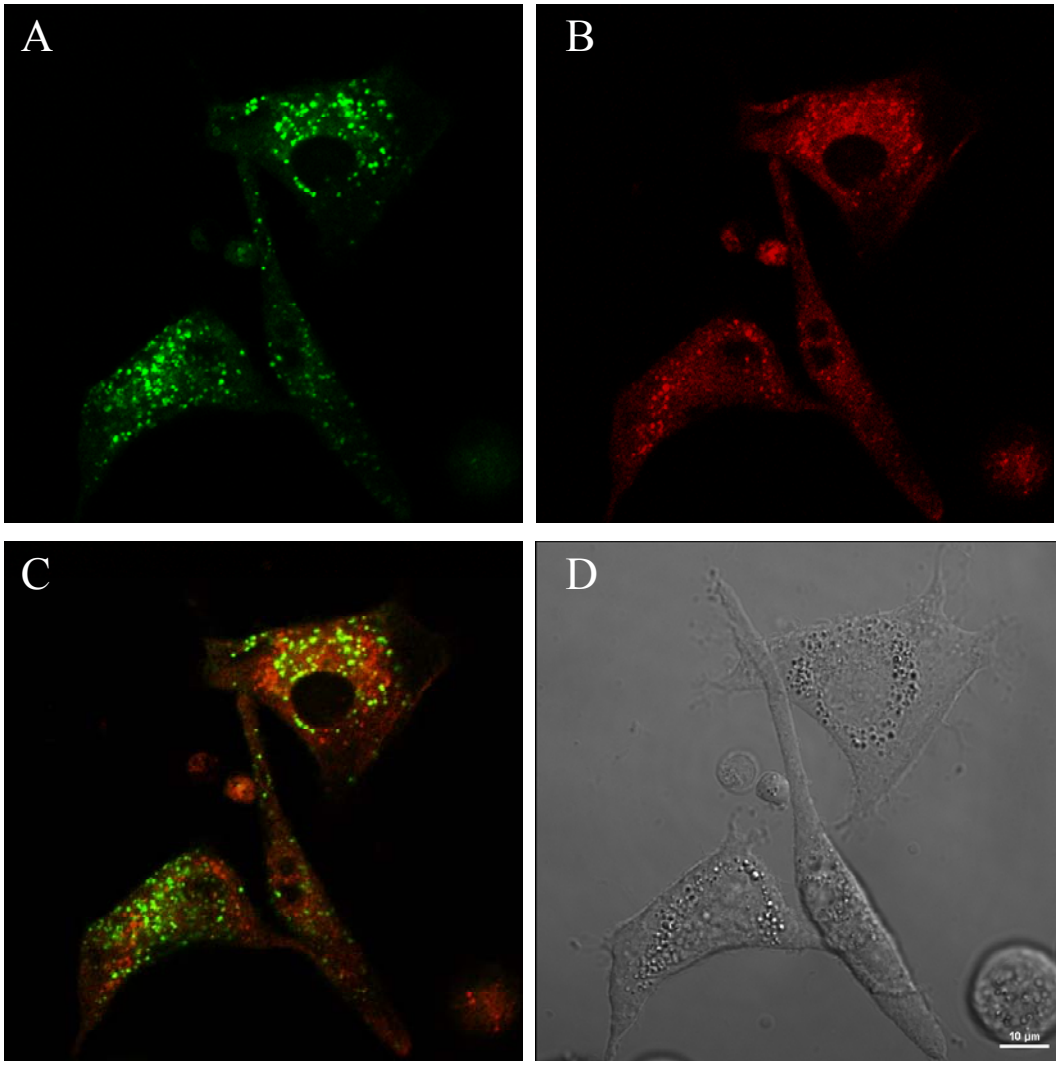
S-Figure 2. KS2 responses to sodium ion, potassium ion, and potassium ion at 140 mM sodium ion condition (a). KS2 responses to sodium ion, calcium ion, magnesium ion, and potassium ion (b).



S-Figure 3. Enlarged figure of KS2 co-stained with Hoechst 33342 (Figure 2c of the manuscript).



S-Figure 4. Confocal fluorescence images of U87MG cells. A depicts the green fluorescence image of cells stained with MitoTracker® Green. B depicts the red fluorescence image of cells stained by KS2. C is the overlay of A and B. D is the bright field image of cells. The yellow color in C indicated the exact colocalization of KS2 with MitoTracker® Green. Mitotracker®Green FM was excited at 488 nm. Its emission was collected using a filter of 515 ± 15 nm. KS2 was excited at 561 nm and its red emission was collected using a filter of 605 ± 37.5 nm. Concentration of KS2 in cell culture medium was 4 μM. Concentration of MitoTracker® Green was 50 nM in the cell culture medium.



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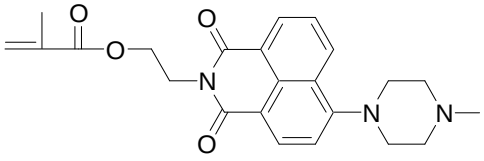
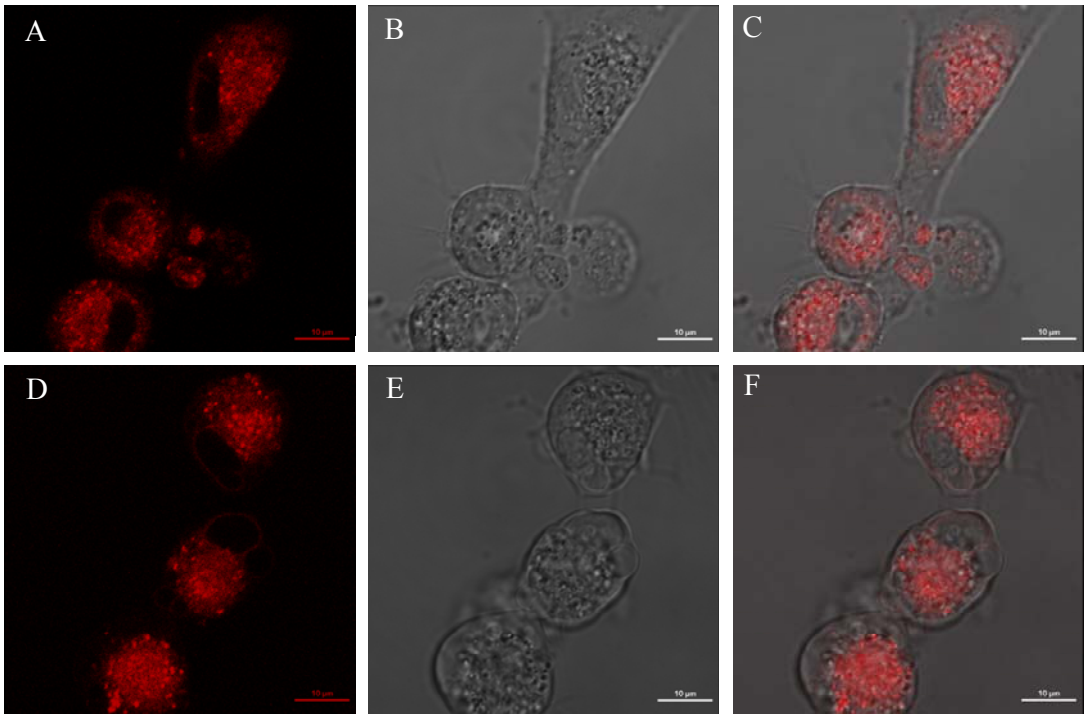
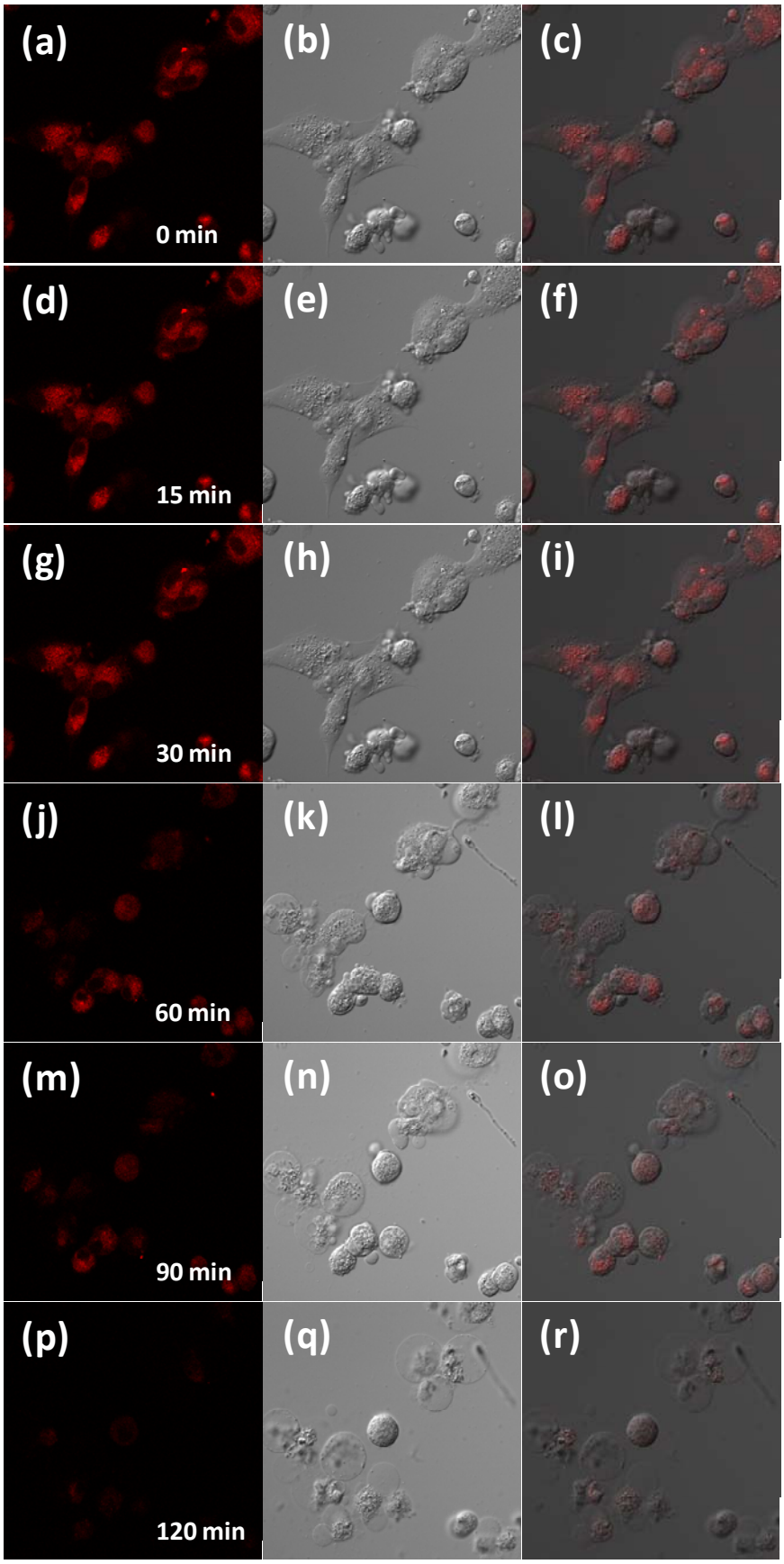


SM3

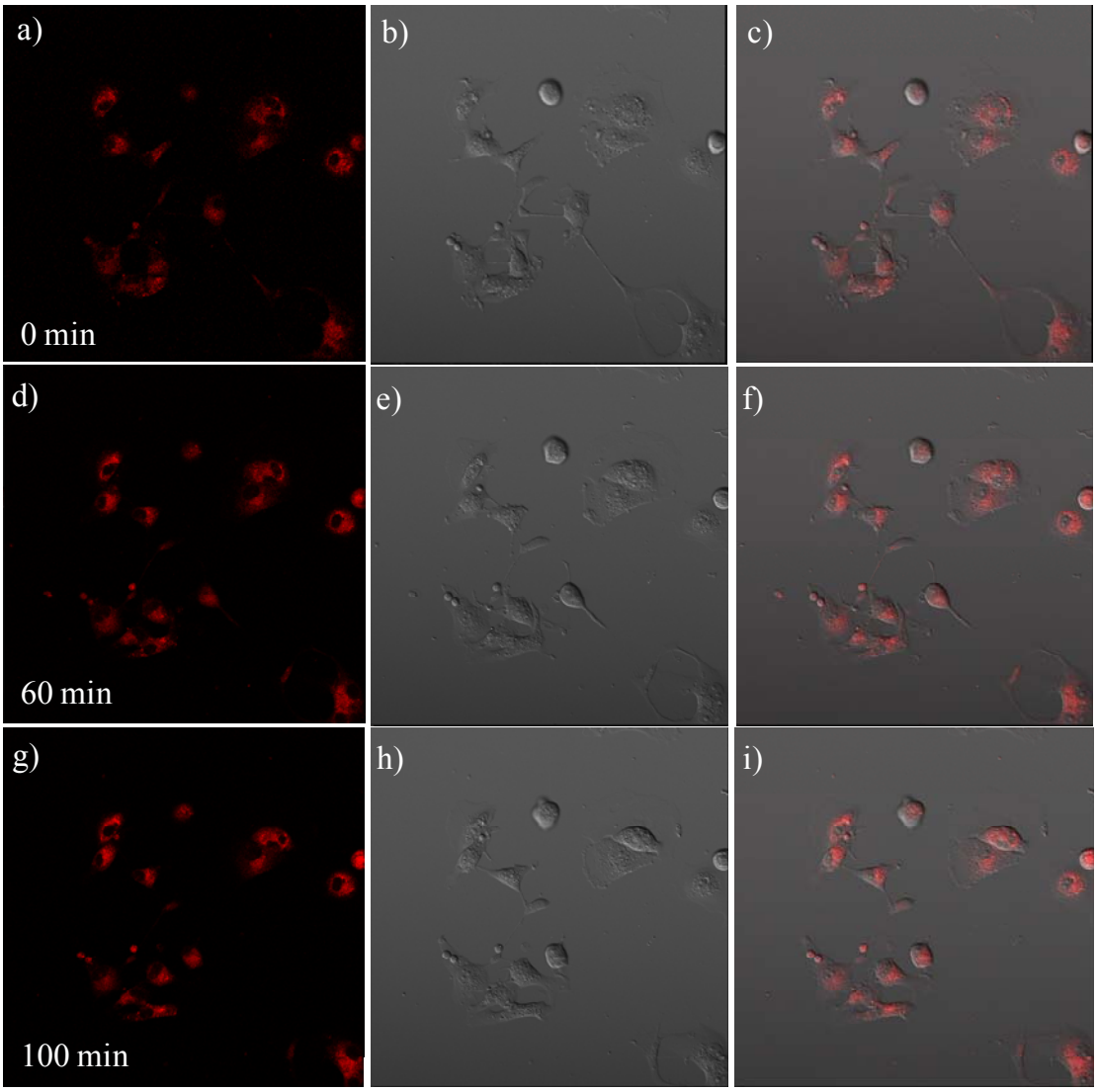
S-Figure 5: Confocal fluorescence images of U87MG cells. A depicts the green fluorescence image of cells stained by SM3. B depicts the red fluorescence image of cells stained by KS2. C is the overlay of A and B. D is the bright field image of cells. E is the chemical structure of SM3. The yellow color in C indicated the exact colocalization of KS2 with SM3. SM3 was excited at 405 nm and its emission was collected using a filter of 515 ± 15 nm. KS2 was excited at 561 nm and its red emission was collected using a filter of 605 ± 37.5 nm. Concentration of KS2 in cell culture medium was 4 µM. Concentration of SM3 was 2 µM in the cell culture medium.



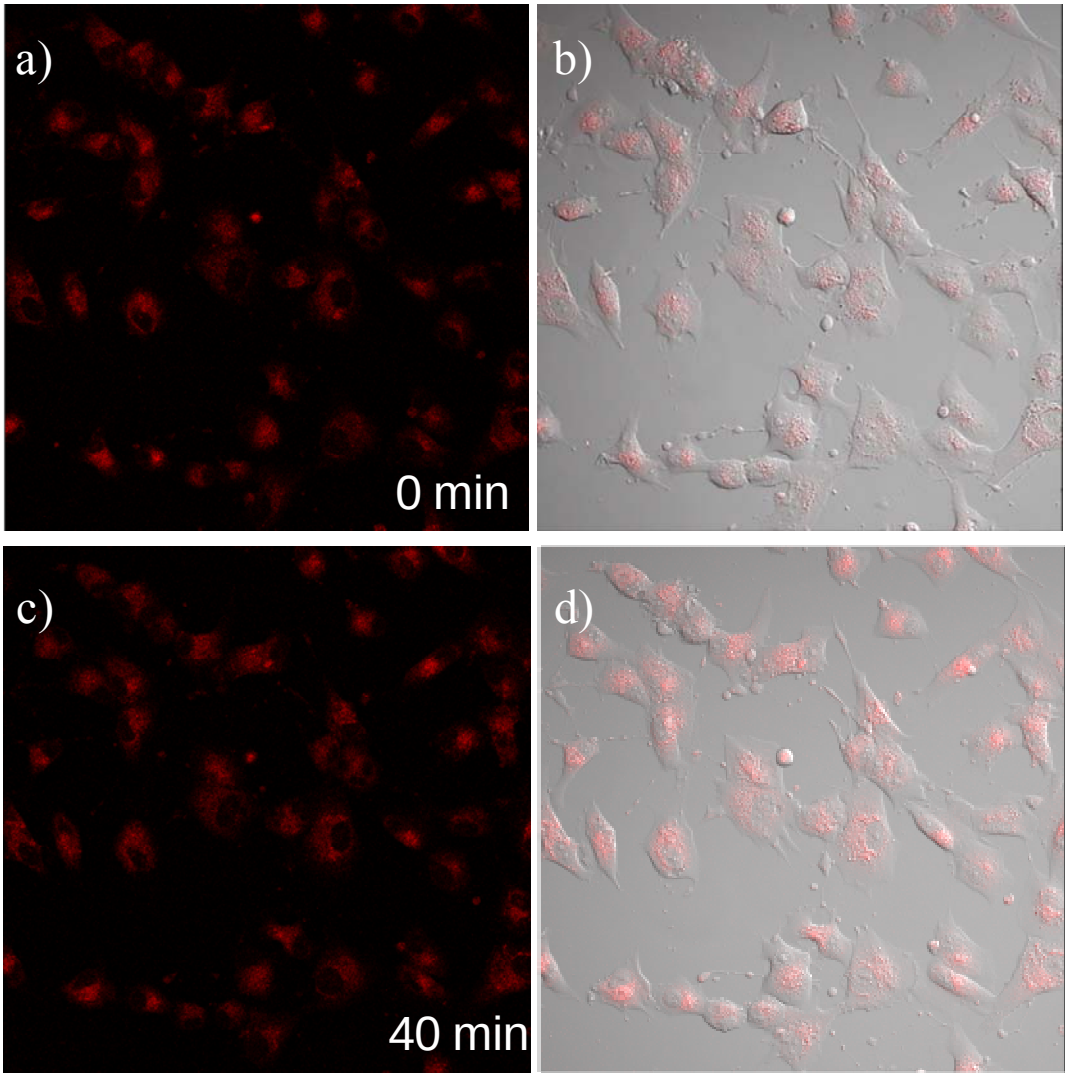
S-Figure 6. Images of U87MG cells stained by KS2 without PBFI (A, B, and C) and with PBFI (D, E, and F). A and D are the fluorescence images. B and E are the bright field images. C is the overlay of A and B. F is the overlay of D and E. PBFI concentration was 10 μM in the cell culture medium. Concentration of KS2 was 2 μM in the cell culture medium. No obvious interference of PBFI on cellular uptake of KS2 was observed at this experimental condition.



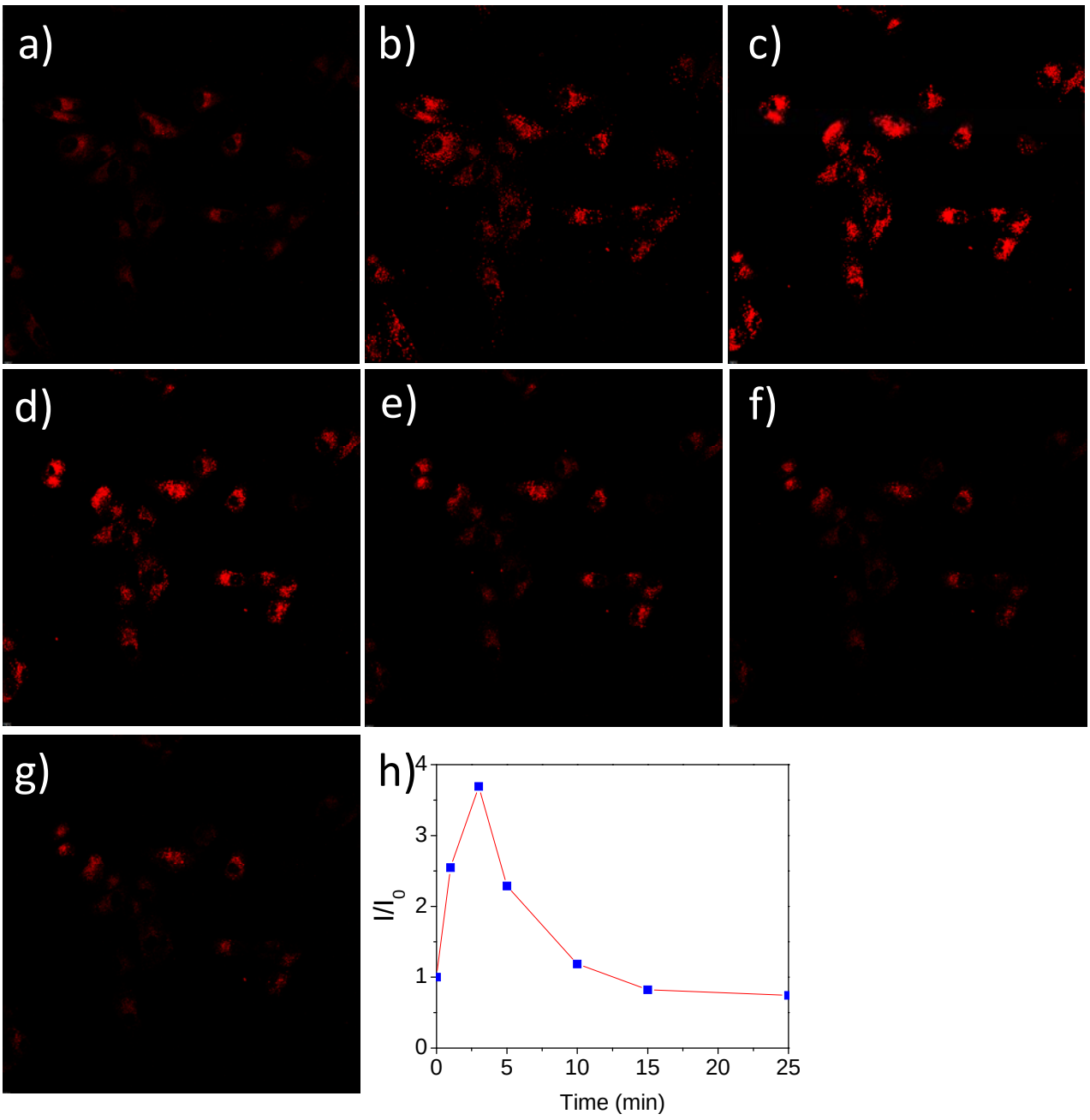
S-Figure 7. Detailed time-course change of intracellular K^+ in U87MG cells after treatment with a mixture of nigericin, bumetanide, and ouabain. Concentration of KS2 was 4 μM. Concentrations of nigericin, bumetanide, and ouabain in the medium were 5 μM, 10 μM, and 10 μM, respectively. Left panel: fluorescence images; middle panel: bright field image; right panel: overlay of fluorescence and bright field images. a, b, and c are for the images at time of 0 before the stimulation. d, e, and f are for the images after 15 minutes drug stimulation. g, h, and i are for the images after 30 minutes drug stimulation. j, k, and l are for the images after 60 minutes drug stimulation. m, n, and o are for the images after 90 minutes drug stimulation. p, q, and r are for the images after 120 minutes drug stimulation. Experimental temperature was 37 °C.



S-Figure 8. Time-course fluorescence of intracellular K^+ in U87MG cells without the treatment of a mixture of nigericin, bumetanide, or ouabain. This is a control experiment for S-Figure 7. Concentration of KS2 was 4 μM. Left panel: fluorescence images; middle panel: bright field image; right panel: overlay of fluorescence and bright field images. a, b, and c are for the images at time of 0. d, e, and f are for the images after 60 minutes. g, h, and i are for the images after 100 minutes. Experimental temperature was 37 °C. Average fluorescence intensity change during the 100 minute time frame is ±5% analyzed using ImageJ.



S-Figure 9. Fluorescence images of KS2 at high concentrated KCl (20 mM) and isoproterenol (5 μM)-containing medium. a and c are fluorescence images. b and d are the fluorescence images superimposed with bright field images. a and b are for time of 0. c and d are for time of 40 minutes. Average fluorescence intensity increased 33% by the isoproterenol stimulation analyzed using ImageJ.



S-Figure 10. Time-dependent fluorescence of U87MG cells stimulated by nigericin observed under confocal fluorescence microscope. a is time of 0 before the addition of nigericin; b, c, d, e, f, and g are time of 1, 3, 5, 10, 15 and 25 minutes, respectively, after the addition of nigericin (20 μM, final concentration) into the 20 mM KCl-containing medium; h gives the average fluorescence intensity ratios measured by ImageJ. I_0 is the average fluorescence intensity from figure a; I is the average fluorescence intensity at different times.

S-Table 1. Quantum efficiencies and extinction coefficients (ε at 560 nm) of the free probe and its complexes at different potassium ion concentrations.

[KCl]	Quantum efficiency*	ε at 560 nm (M^{-1} cm^{-1})
0 mM	0.11%	38400
140 mM	0.52%	35300
1400 mM	5.6%	34500

* Low quantum efficiency of a TCF derived fluorophore in solution (ethanol) was reported earlier by Lord et al (*J. Am. Chem. Soc*. 2008, 130, 9204-9205, ref 12 in manuscript) to be 2.5%.