Electronic Supplementary Information for

A dual-fluorophore sensor approach for ratiometric fluorescence imaging of potassium in living cells

Zeming Wang, [1] *Tyler C. Detomasi,* [1] *Christopher J. Chang*[1,2,3]*

[1]Department of Chemistry, University of California, Berkeley, California 94720, United States
[2]Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, United States
[3]Helen Wills Neuroscience Institute, University of California, Berkeley, California 94720, United States

*Corresponding author: chrischang@berkeley.edu

Table of Contents

Experimental Details

General Methods, Cell Culture procedures, and **Confocal Fluorescence Imaging Experiments** are described in the main text.

Quantum Yield. Quantum yield values of PS525 were calculated using the equation $\phi_{sample} = \phi_{standard}$ $(Grad_{sample}/ Grad_{standard})(\eta_{sample}^2 / \eta_{standard}^2)$. Rhodamine 101 prepared in ethanol was used as a standard for Coumarin 343, where $\phi_{standard} = 1.00$. Rhodamine B prepared in water was used as a standard for PS525, where $\phi_{standard} = 0.31$.[1] Quantum yields of PS525, Coumarin 343, RPS-1 in both 0 mM and 200 mM K^+ buffer (50 mM HEPES, pH=7.4) were determined.

Dissociation Constant of RPS-1. Dissociation constant (K_d) of the probe is calculated using a variation of Benesi-Hildebrand plot,[2] derived from the following equations.

$$F = F_P + F_C = \emptyset_P \varepsilon_P[P] + \emptyset_C \varepsilon_C[C]$$

$$K_d = \frac{[K^+][P]}{[C]}$$

Here F is the fluorescence intensity, Ø is the quantum yield, ε is the extinction coefficient, P is unbound form of RPS-1, and C is K^+-bound RPS-1.

$$F_0 = \emptyset_P \varepsilon_P[P_0]$$

$$[P_0] = [P] + [C]$$

$$\frac{F_0}{F - F_0} = \left(\frac{\emptyset_P \varepsilon_P}{\emptyset_P \varepsilon_P - \emptyset_C \varepsilon_C}\right) K_d \frac{1}{[K^+]} + \left(\frac{\emptyset_P \varepsilon_P}{\emptyset_P \varepsilon_P - \emptyset_C \varepsilon_C}\right)$$

5 μM RPS-1 was added to HEPES buffer (50 mM, pH =7 .4) containing 5, 10, 15, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 mM K^+. Fluorescence intensities of each sample was recoded and plotted against inverse value of K^+ concentration. K_d was determined using a linear fitting analysis.

pH sensitivity Test. Fluorescence spectra at the following pH values and indicated buffers were obtained for both PS525 and Coumarin 343: 50 mM sodium acetate (5.0, 5.5), 50 mM MES (6.0, 6.5), 50 mM HEPES (7.0, 7.5, 8.0). All buffers are K^+ free buffers. PS525 was measured at 3 μM and Coumarin 343 was measured at 5 μM

In Vitro **Characterization RPS-1 Esterase Catalyzed Hydrolysis Dynamics.** A 5 µL aliquot of porcine liver esterase (Sigma-Aldrich) was added to a falcon tube containing 5 mL of pH 7.4 HEPES buffer and 10 μM RPS-1. The tube was shaken vigorously and incubated at 37 °C water

bath for 3 h. 150 μL samples were taken every 10 min and quenched with 50 μL 1% trifluoroacetic acid solution. The samples were then filtered and analyzed using LC-MS. The relative amount of each integrated peak for PS525 and RPS-1 were used for the analysis.

***In Vitro* Characterization of Esterase-Treated RPS-1.** 5 μM RPS-1 was dissolved in 1 mL HEPES buffer (50 mM, pH = 7.4, no K^+). Fluorescence spectrum was acquired with excitation first at 405 nm and then at 525 nm. Then, 1 μL porcine liver esterase solution (10 mg/mL in 3.2 M $(NH_4)_2SO_4$) was added and combined solution was incubated at 37 °C for 30 min.[3] Fluorescence spectra were recorded as before. KCl solution was added to the buffer until $[K^+]$ = 200 mM. Fluorescence spectra were recorded again as before.

***In Vitro* Limit of Detection (LOD) Test of Ratiometric Fluorescent Response.** 5 μM PS525 and 5 μM Coumarin 343 were added to HEPES buffer (50 mM, pH =7 .4) containing 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 mM K^+. Fluorescence intensities of each sample were recoded. Peak intensities at both 551 nm and 490 nm were used to calculated ratio intensity and plotted against inverse value of K^+ concentration. Linear fitting was conducted on the final plot. LOD was calculated by LOD = 3.3 * σ / S where S is the slope of the calibration curve and σ is the standard deviation of the regression line.

RPS-1 Cellular Trappability Experiments. HeLa cells were plated in phenol red-free medium on borosilicate chamber slides (Nunc) and allowed to grow to 60% confluence 1 d prior to imaging. Cells were stained by incubating a 10 μM solution of RPS-1 in DMEM for 30 min. DMEM media was then aspirated from each chamber containing cells and replaced with RPS-1 free DMEM media. Snapshot images were taken. Following, each chamber was washed 3 times with RPS-1 free DMEM media and snapshot images were taken after each wash with both 458 nm and 514 nm excitation.

Flow Cytometric Analysis of Valinomycin-Treated HeLa Cell Viability using Propidium Iodide (PI) Staining. Cells were plated in 12-well polystyrene culture plates (Corning). Cell culture media was aspirated and DMEM media containing 10% FBS and the solution with or without 10 μM Valinomycin was added in chambers containing cells and incubated at 37 °C for 1 h. After incubation was complete, media was removed and 900 μL PBS (0 mM K^+) containing 3 μM propidium iodide (PI) was added to each well. Cells were dislodged by agitation and filtered through 35 μm nilon mesh caps into a 12 x 75 mm polystyrene tube (corning) for analysis by flow cytometry. Finally, cell viability was calculated as the percentage of PI negative cells.

Astemizole Induced K^+ Influx Experiments. HT29, HeLa, A549 cells were plated in phenol red-free medium on borosilicate chamber slides (Nunc) and allowed to grow to 60% confluence 1 d prior to imaging. Cells were stained by incubating a 10 μM solution of RPS-1 in DMEM at

37 °C for 1 h. Cells were washed one time with RPS-1 free DMEM media. Cell media was then exchanged to high K^+ buffer (50 mM HEPES, pH = 7.4, 40 mM K^+, 100 mM Na^+) with or without 5 μM astemizole and incubated at 37 °C for 1 h. Images were taken after incubation for each cell lines.

Flow Cytometric Analysis of RPS-1 Treated Cell Viability using Propidium Iodide (PI) Staining. MCF-10A, MDAMB468, MDAMB231, U2OS, RKO, PC-3 cells were plated in 12-well polystyrene culture plates (Corning) and allowed to grow to 70% confluence. Cells were stained by incubating a 10 μM solution of RPS-1 in DMEM at 37 °C for 2 h. After incubation was complete, media was removed and 900 μL PBS containing 3 μM propidium iodide (PI) was added to each well. Cells were dislodged by agitation and filtered through 35 μm nilon mesh caps into a 12 x 75 mm polystyrene tube (corning) for analysis by flow cytometry. Finally, cell viability was calculated as the percentage of PI negative cells.

Ratio Image Analysis. Ratio images were generated using Ratio Plus plugin in ImageJ and parameters are set as following. Background for green channel is at 40 and background for blue channel is at 80 for visualization. Multiplication factor was set at 1 and clipping value for both channels was set at 0. All quantification of images was done after generation of ratio images.

Supplementary Figures

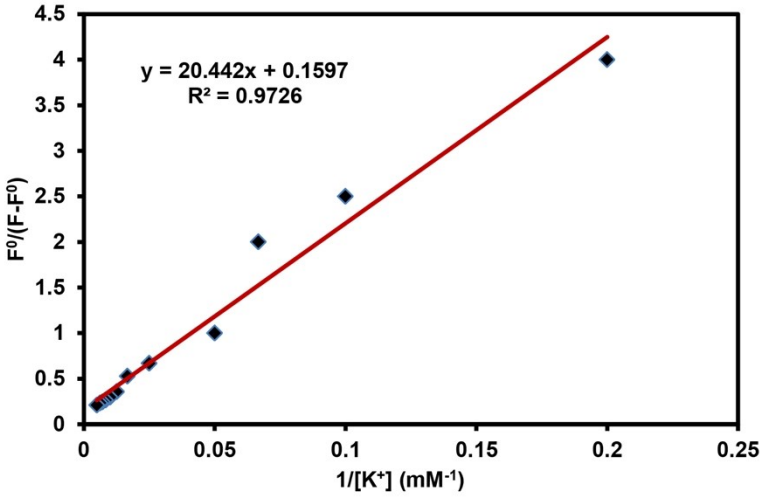

Figure S1. Determination of potassium dissociation constant of PS525. K_d = 128 mM.

	ε (M^{-1} cm-1)	λ_{max} (nm) absorbance	Φ_{PS525}	$\Phi_{Coumarin}$
PS525	61100	525	0.03	
PS525 + K^+	65000	524	0.17	
Coumarin 343	42100	445		0.02
Coumarin 343 + K^+	44900	443		0.02
RPS-1	26000	451		0.01
	32567	526	0.03	
RPS-1 + K^+	26333	449		0.01
	34800	522	0.15	

Figure S2. Tabulated data on extinction coefficient and quantum yield values for PS525, Coumarin 343, and RPS-1 in 0 mM and 200 mM K^+ HEPES buffer (50 mM, pH = 7.4).

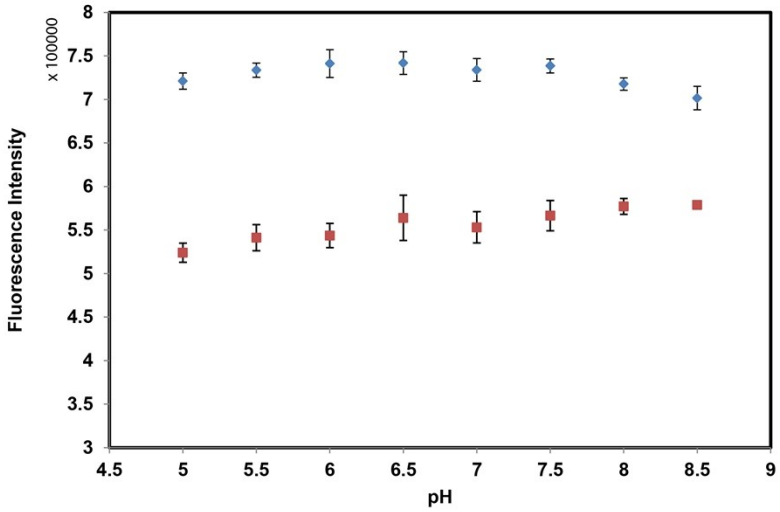

Figure S3. Fluorescence spectra of Coumarin 343 (red) and PS525 (blue) at the following pH values and indicated buffers: 50 mM sodium acetate (5.0, 5.5), 50 mM MES (6.0, 6.5), 50 mM HEPES (7.0, 7.5, 8.0). Coumarin 343 was excited at 450 nm and PS525 was excited at 525 nm. Peak intensity value for each spectrum was obtained and plotted. Error bars denote SEM, n=3.

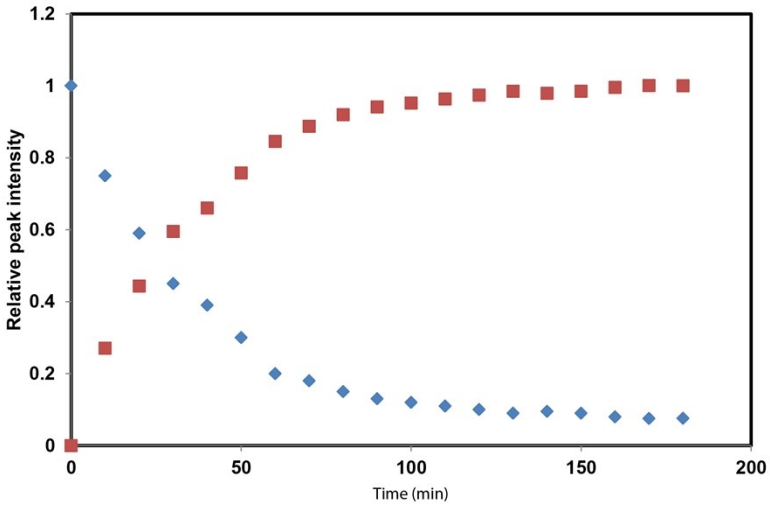

Figure S4. Time-dependent changes in PS525 (red) and RPS-1 (blue) within 3 h upon treatment with porcine liver esterase 37.5 nM. RPS-1 were incubated at 37 °C in HEPES buffer (pH 7.4) with porcine liver esterase. Relative peak intensities were integrated and calculated through LC-MS chromatography.

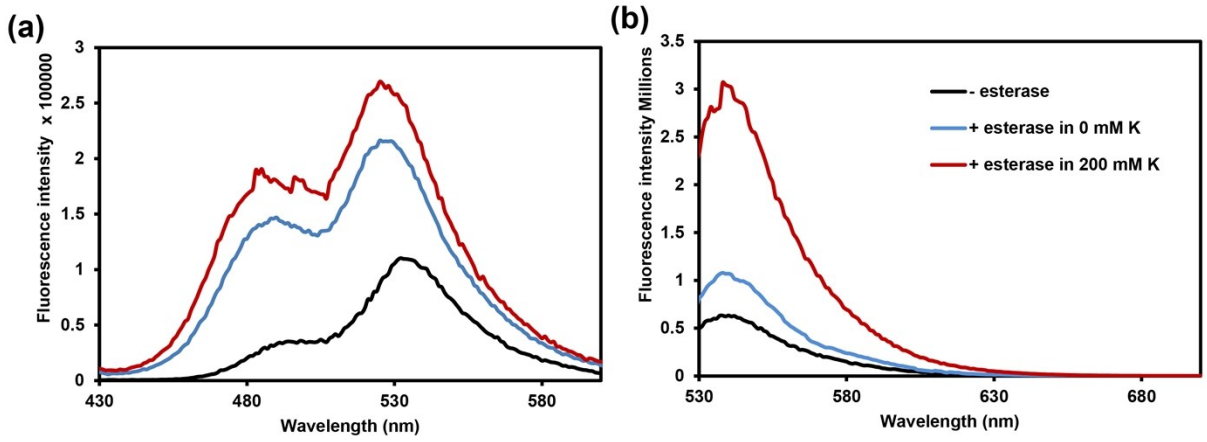

Figure S5. (a) RPS-1 emission spectrum with 405 nm excitation. (b) RPS-1 emission spectrum with 525 nm excitation. Measurements were taken before addition of porcine liver esterase (black), after addition of porcine liver esterase and before addition of K^+ (blue), and after addition of K^+ (red).

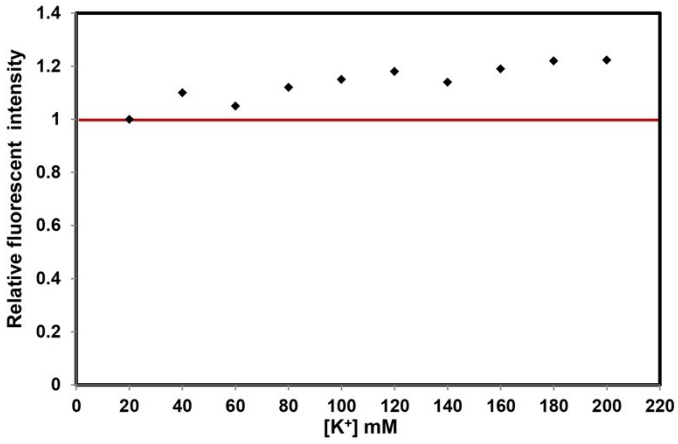

Figure S6. Coumarin 343 fluorescence intensities over varying [K^+], showing that this dye is minimally responsive to changes in K^+ levels. Measurements were made with 50 mM HEPES buffer (pH 7.4) and 5 μM Coumarin 343.

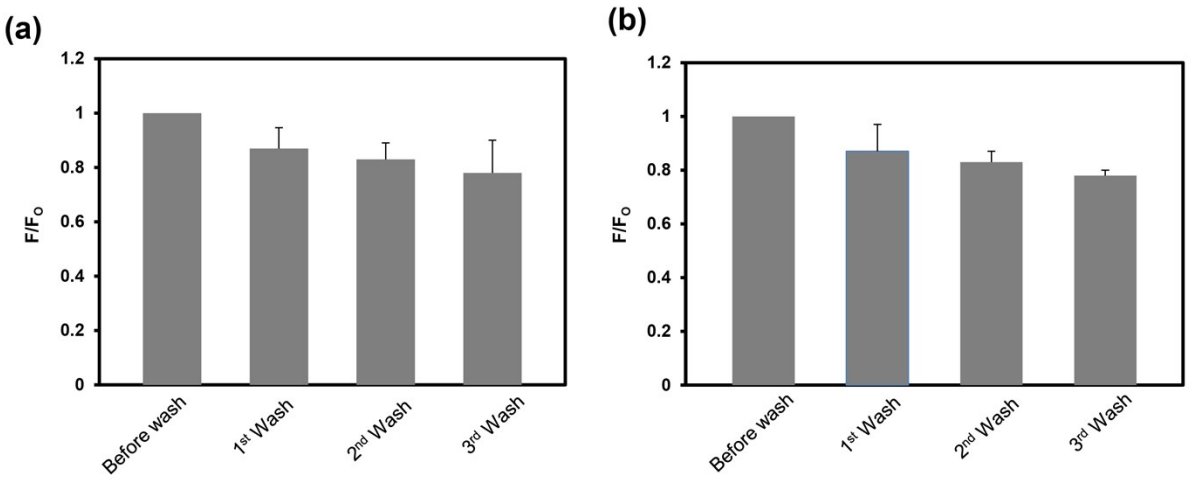

Figure S7. Cell trappability analysis of RPS-1. HeLa cells were treated with 10 μM RPS-1 and washed with RPS-1-free DMEM media 3 times. Images were acquired after each wash. (a) Excitation at 458 nm, Blue Channel. (b) Excitation at 514 nm, Green Channel. Error bars denote SEM, n=3.

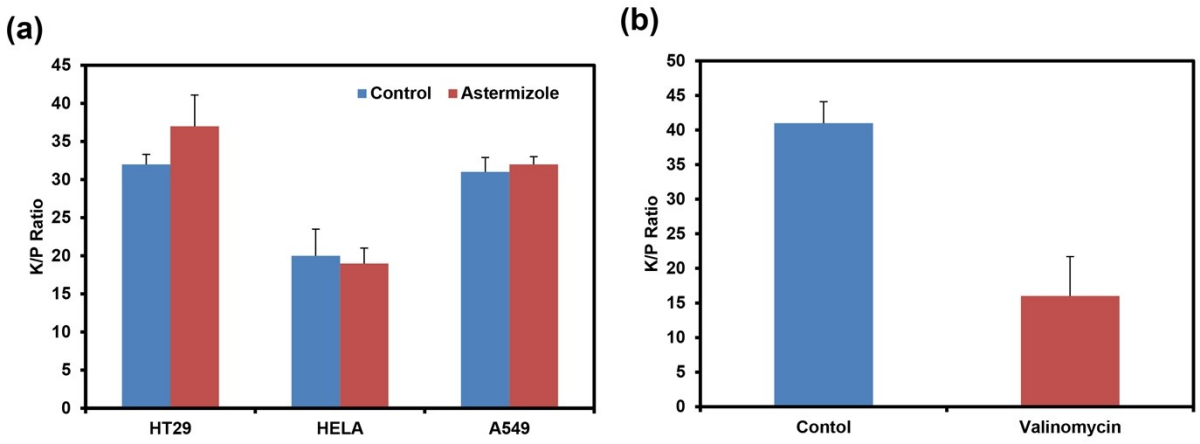

Figure S8. Total potassium content of control versus valinomycin- or astermizole-treated cells measured using ICP-MS. (a) HT29, HeLa, A549 cells were incubated with high K^+ buffer (50 mM HEPES, pH = 7.4, 40 mM K^+, 100 mM Na^+) with or without 5 μM astemizole and incubated at 37 °C for 1 h and then washed with cold K^+ free PBS buffer. (b) HeLa cells were incubated with or without 10 μM valinomycin for 1 h and then washed with cold K^+ free PBS buffer. Cellular potassium and phosphorous content was measured by ICP-MS. Error bars denote SEM, n=3.

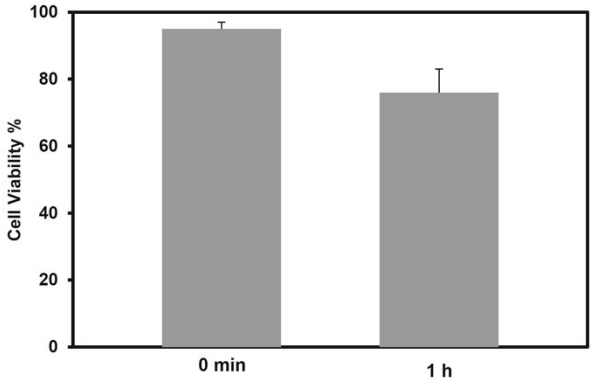

Figure S9. Cell viability of HeLa cells upon valinomycin treatment. HeLa cells were incubated with 10 μM valinomycin at 37 °C for 1 h. Cell viability was measured by propidium iodide (PI assay). Error bars denote SEM, n=3.

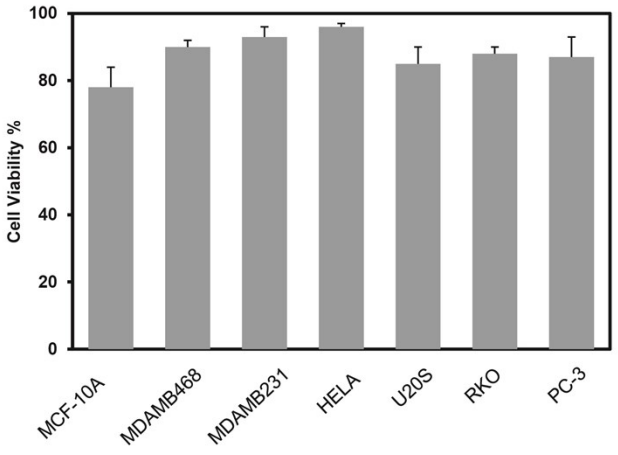

Figure S10. Cell viability of MCF-10A, MDAMB468, MDAMB231, U2OS, RKO, PC-3 cells upon RPS-1 staining. Cells incubated with a 10 μM solution of RPS-1 in DMEM at 37 °C for 2 h. Cell viability was measured by propidium iodide (PI assay). Error bars denote SEM, n=3.

References

1 D. Magde, G. E. Rojas and P. G. Seybold, *Photochem. Photobiol.*, 1999, **70**, 737–744.

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