ACS APPLIED MATERIALS & INTERFACES

Letter

www.acsami.org

Near-IR Two-Photon Fluorescent Sensor for K^+ Imaging in Live Cells

Binglin Sui,[†] Xiling Yue,[†] Bosung Kim,[†] and Kevin D. Belfield*[,†,‡,§]

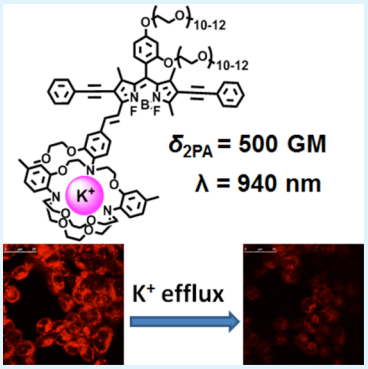
[†]Department of Chemistry, University of Central Florida, Orlando, Florida 32816, United States

[‡]College of Science and Liberal Arts, New Jersey Institute of Technology, Newark, New Jersey 07102, United States

[§]School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, P.R. China

Supporting Information

ABSTRACT: A new two-photon excited fluorescent K^+ sensor is reported. The sensor comprises three moieties, a highly selective K^+ chelator as the K^+ recognition unit, a boron-dipyrromethene (BODIPY) derivative modified with phenylethynyl groups as the fluorophore, and two polyethylene glycol chains to afford water solubility. The sensor displays very high selectivity (>52-fold) in detecting K^+ over other physiological metal cations. Upon binding K^+, the sensor switches from nonfluorescent to highly fluorescent, emitting red to near-IR (NIR) fluorescence. The sensor exhibited a good two-photon absorption cross section, 500 GM at 940 nm. Moreover, it is not sensitive to pH in the physiological pH range. Time-dependent cell imaging studies via both one- and two-photon fluorescence microscopy demonstrate that the sensor is suitable for dynamic K^+ sensing in living cells.



KEYWORDS: *potassium sensors, fluorescent sensors, two-photon absorption, two-photon fluorescence microscopy, cell imaging*

It is difficult to overstate the physiological importance of potassium for life, as its indispensable roles in a variety of biological processes are widely known,[1−4] e.g., a number of human diseases have been found to be related to abnormal potassium levels in the body.[5−8] As a result, efficient methods for determining physiological levels of potassium are of paramount importance. To this end, numerous potassium sensors have been designed and synthesized. Among these sensors, fluorescent sensors have attracted significant attention because of the outstanding merits of fluorescence techniques, such as high sensitivity, convenience, fast response, and noninvasiveness.

However, the only commercially available fluorescent potassium sensor, potassium-binding benzofuran isophthalate (PBFI), suffers insufficient potassium binding strength and poor selectivity toward K^+ over Na^+.[9] In recent years, fluorescent sensors incorporating the 2-triazacryptand [2,2,3]-1-(2-methoxyethoxy)benzene (TAC) group as the K^+-sensing moiety have been prepared and applied to biological imaging.[10−14] TAC demonstrates very high selectivity in detecting K^+ over other physiological metal cations, and to date is the best K^+-selective chelator.

Over the past two decades, two-photon fluorescence microscopy (2PFM) underwent considerable development.[15,16] Compared with one-photon fluorescence microscopy, 2PFM has the advantages of localized excitation, increased penetration depth, reduced phototoxicity, prolonged observation time, and efficient light detection.[17,18] With the rapid development of two-photon excited fluorescence techniques, two-photon fluorescent sensors have been exploited to determine various metal cations in physiological environments, including Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^+.[19−23] To the best of our knowledge, no two-photon excited fluorescent K^+ sensor has been reported thus far.

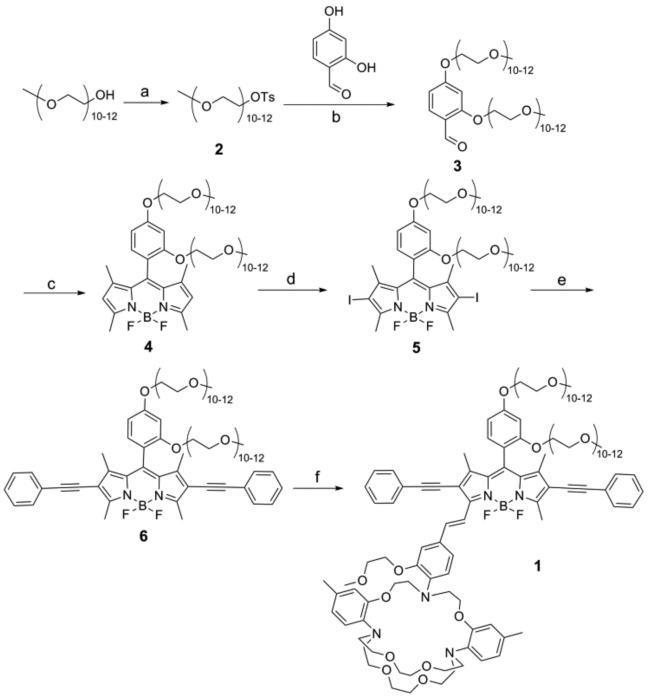
Herein, we report an efficient two-photon fluorescent sensor for K^+ ions. This new K^+ sensor (**1**) is established on a BODIPY platform and the TAC group. The TAC group was attached to the BODIPY core via a double bond similar to a reported K^+ sensor.[12] This condensation reaction has been well studied and resulting adducts applied to sensing metal cations in recent years.[24,25] Two long polyethylene glycol (PEG) chains were introduced to the BODIPY core to increase the water solubility of the dye. Two phenylethynyl groups were conjugated to the BODIPY core to extend the extent of conjugation of the chromophore and, hence, increase its two-photon absorption (2PA) cross-section, making it an efficient two-photon excited fluorescent K^+ sensor.

The synthetic route for the preparation of sensor **1** is shown in Scheme 1. The synthesis of **1** began with PEG 550, which was used to prepare tosylated PEG **2** according to a literature procedure.[26] Then compound **2** was reacted with 2,4-dihydroxybenzaldehyde in the presence of potassium carbonate to produce benzaldeyde **3**. Compound **4** was synthesized via a condensation reaction of **3** with 2,4-dimethylpyrrole in the presence of a catalytic amount of trifluoroacetic acid (TFA) at room temperature, followed by an oxidation reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and a subse-

Received: May 24, 2015
Accepted: August 5, 2015
Published: August 10, 2015

DOI: 10.1021/acsami.5b04506
ACS Appl. Mater. Interfaces 2015, 7, 17565−17568

Scheme 1. Synthetic Route of Sensor 1[a]



[a]Reagents and conditions: (a) TsCl, NaOH, THF, H_2O, 0 °C, 99.0%; (b) K_2CO_3, DMF, 130 °C, overnight, 89.0%; (c) 2,4-dimethylpyrrole, TFA, CH_2Cl_2, room temperature, overnight; then DDQ, 4 h; then TEA, $BF_3{\cdot}OEt_2$, overnight, 21.5%; (d) NIS, CH_2Cl_2, in the dark, room temperature, 5 h, 71.2%; (e) phenylacetylene, CuI, $Pd(PPh_3)_4$, THF:TEA (4:1), in the dark, 45 °C, 16 h, 77.1%; (f) TAC−CHO, piperidine, glacial acetic acid, *n*-butanol:toluene (1:1), reflux, overnight, 10.3%.

quent chelation reaction with $BF_3{\cdot}OEt_2$ in the presence of triethylamine (TEA). Iodination of **4** with N-iodosuccinimide (NIS) in the dark afforded **5** as a red liquid. Sonogashira coupling reaction was performed between **5** and phenylacetylene with $Pd(PPh_3)_4$ and CuI as the catalysts in 4:1 THF:TEA to provide penultimate intermediate **6** as a purple liquid. The target product (**1**) was obtained through a Knoevenagel condensation reaction between **6** and TAC-aldehyde, which was prepared according to a literature procedure,[10] in the presence of piperidine and glacial acetic acid in 1:1 *n*-butanol:toluene.

The K^+ sensing ability of sensor **1** was examined in 30 mM HEPES buffer (pH 7.2)/MeCN (9:1, v/v). The fluorescence emission spectra of **1** in the presence of various biologically important metal cations at their physiological concentrations are shown in Figure 1a. The counter ion for all metal cations used in this study was chloride. Free **1** was virtually nonfluorescent in the absence of cations (quantum yield: 0.001). Upon the addition of K^+, red-NIR fluorescence with a maximum emission wavelength at 644 nm was immediately observed. However, the addition of other metal cations brought about no considerable change to the fluorescence spectrum of **1**. Therefore, sensor **1** exhibited high selectivity for K^+ over other biologically important cations.

K^+ titration experiments were carried out to further investigate the efficiency of **1** toward K^+ sensing in 30 mM HEPES buffer (pH 7.2)/MeCN (9:1, v/v) solutions. To maintain a constant ionic strength, sample solutions were balanced with NaCl. UV−vis absorption spectroscopy was first

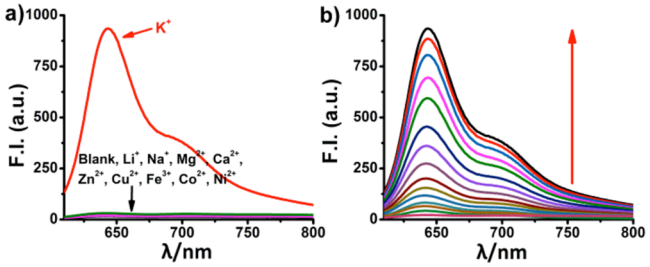


Figure 1. Fluorescence emission spectra of **1** (1 μM) in 30 mM HEPES buffer (pH 7.2)/MeCN (9:1, v/v), (a) upon addition of various biologically important metal cations at their physiological concentrations: Li^+ (1.0 mM), Na^+ (150 mM), K^+ (150 mM), Mg^{2+} (2.0 mM), Ca^{2+} (2.0 mM), Zn^{2+} (50 μM), Cu^{2+} (50 μM), Fe^{3+} (50 μM), Co^{2+} (50 μM), Ni^{2+} (50 μM); and (b) upon progressive addition of K^+. The counter ion for all metal cations was chloride.

used to conduct titration experiments. As displayed in Figure S1, the solution of **1**, in the absence of K^+, exhibited absorption at 637 nm. Upon the progressive addition of K^+, the absorption maximum of **1** gradually underwent a hypsochromic shift to 617 nm. With the absorption wavelength change from 637 to 617 nm the color of the sample showed a slight visible change before and after the addition of K^+ by naked eye examination since both absorption maxima correspond to greenish blue color. Simultaneously, the absorption intensity of the sample moderately increased. Such changes in the absorption spectrum of **1** in response to the interaction with K^+, which are general phenomena of amine-containing BODIPY derivatives and other fluorophores, have been attributed to the suppression of internal charge transfer (ICT).[12,25] Fluorescence emission titration experiments were performed to examine the K^+ sensing ability of sensor **1**. Figure 1b shows the fluorescence emission spectra of **1** in the absence and presence of different concentrations of K^+ ions. With the concentration of added K^+ increasing, the fluorescence emission intensity was enhanced accordingly (see Figure S2). In the presence of 150 mM K^+, the fluorescence intensity of **1** increased 52-fold (quantum yield: 0.36). The dissociation constant (K_d) of the sensor was determined to be 71 mM (see the Supporting Information), according to a literature method.[13] The linear fitting in Figure S3 evidenced 1:1 complexation behavior of sensor **1**.[13] The detection limit of **1** for K^+ was determined to be 5.4×10^{-7} M, which is suitable for detecting physiological K^+ levels (5−150 mM).[13]

In addition, the pH sensitivity of **1** was investigated as a function of pH between 3.0 and 8.0 in 9:1 (v/v) H_2O/MeCN solution. Figure S4 shows the fluorescence emission spectra of **1** at various pH. In the solutions of **1** with pH values between 6.0 and 8.0, no considerable fluorescence emission signal was detected. However, as the pH decreased below 6.0, the fluorescence emission intensity of **1** became stronger. These results suggest that sensor **1** is not pH-dependent in typical physiological environments, where the pH is generally in the range of 6.8−7.4.

Moreover, two-photon absorption (2PA) experiments were carried out to measure the 2PA cross-section of **1**. The 2PA properties of BODIPY dyes have been described in literature.[27,28] The open-aperture Z-scan method[29] was used for direct 2PA cross-section measurements over a broad spectral region, 940−1260 nm, with a single laser beam as the irradiation light source. Figure 2 shows the one-photon and two-photon absorption spectra of sensor **1**. The linear or one-

DOI: 10.1021/acsami.5b04506
ACS Appl. Mater. Interfaces 2015, 7, 17565−17568

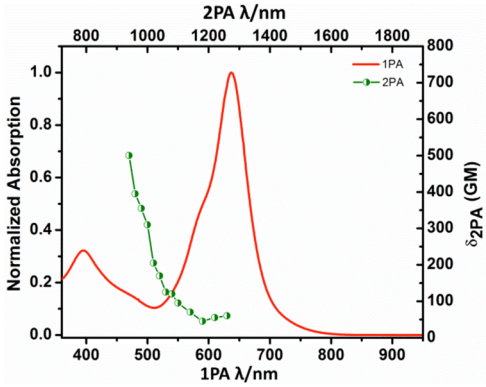


Figure 2. One-photon (linear) absorption (1PA) (red solid) and two-photon absorption (2PA) (green circles) spectra of **1**.

photon absorption (1PA) maximum was 637 nm. Sensor **1** exhibited a maximum 2PA cross section (δ_{2PA}) of 500 GM [1 GM (Göpper Meyer) = 1×10^{-50} cm^4 s photon^{-1}] at 940 nm. Compared to previous reports for 2PA fluorescent probes for other metal cations (in which 2PA cross-sections are generally around 100 GM),[19−23] the high 2PA cross section, long absorption wavelength, and red-NIR fluorescence emission of sensor **1** make it a highly promising 2PA fluorescent K^+ sensor for 2PFM.

On the basis of the desirable properties of sensor **1** in sensing K^+, it was applied to detect changes in the concentration of K^+ in living cells. Both one-photon and two-photon fluorescence microscopy were used to investigate the fluorescence intensity changes of the sensor in response to the K^+ efflux in living HCT-116 cells (see experimental details in the Supporting Information). Cell viability tests showed that cells exhibited no considerable decrease in viability through 6 h after incubation with 5 μM of sensor **1** (see Figure S5). Figure 3 shows time-dependent one-photon fluorescence microscopy images of HCT-116 cells. Cells incubated with sensor **1** were highly fluorescent as shown in images a and c, which is consistent with intracellular K^+ levels (around 150 mM) high enough to trigger strong fluorescence of **1**.

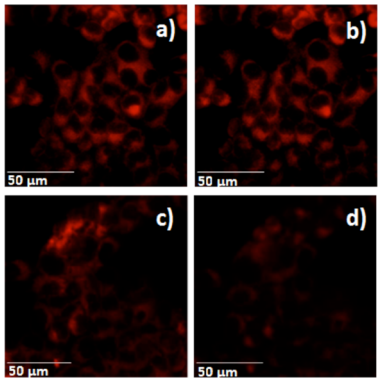


Figure 3. One-photon (conventional) fluorescence microscopy images of live HCT-116 cells incubated only with **1** at (a) 0 and (b) 30 min; and HCT-116 cells incubated with **1** and a combination of nigericin, ouabain octahydrate, and bumetanide at (c) 0 and (d) 30 min. The scale bar is 50 μm.

It has been shown that a combination of nigericin, ouabain octahydrate, and bumetanide can effectively bring about K^+ efflux of cells.[13,30] To examine the ability of the sensor to respond to K^+ level changes in living cells, we incubated cells with a combination of nigericin, ouabain octahydrate, and bumetanide, and then imaged at different times (Figure 3). With K^+ depletion induced by nigericin, ouabain octahydrate, and bumetanide, the fluorescence intensity from cells gradually decreased (from image c to d). As a control, cells without treatment with these agents remained highly fluorescent (Figure 3b), exhibiting almost no visible change compared to their original state (Figure 3a). To quantitatively illustrate the fluorescence changes of cells, the average fluorescence emission intensity of cell images was determined at different times and shown in Figure S6. The significant difference between the two curves that illustrate cell fluorescence intensity changes over time demonstrated the ability of sensor **1** in sensing intracellular K^+ levels.

The relatively high 2PA cross-section of probe **1** motivated us to explore two-photon fluorescence microscopy to further study the K^+ sensing of **1** in living HCT-116 cells, following the same experimental procedures as employed in one-photon fluorescence microscopy. As shown in Figure 4, for cells incubated only with sensor **1**, serving as the control group, little visible change was observed between live cell images taken at 0 min (image a) and 30 min (image b).

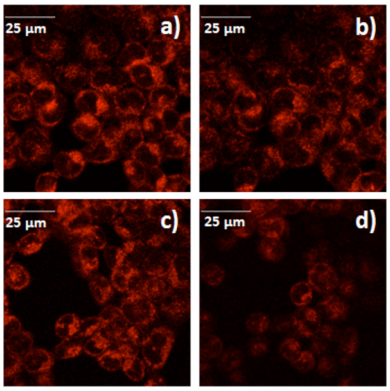


Figure 4. Two-photon fluorescence microscopy images of live HCT-116 cells incubated only with **1** at (a) 0 and (b) 30 min; and cells incubated with **1** and a combination of nigericin, ouabain octahydrate, and bumetanide at (c) 0 and (d) 30 min. The scale bar is 25 μm.

However, a significant decrease in fluorescence intensity of cells was observed in Figure 4c (0 min) to Figure 4d (30 min) when cells were incubated with sensor **1** and a combination of nigericin, ouabain octahydrate, and bumetanide. Fluorescence intensity data of cells at different time points quantitatively demonstrated the fluorescence signal change of cells (see Figure S7). Cells treated with nigericin, ouabain octahydrate, and bumetanide displayed gradually decreased fluorescence with incubation time due to K^+ efflux (curve B in Figure S7). By contrast, fluorescence from cells without incubation with these agents remained relatively constant (curve A in Figure S7). These data are in good agreement with results obtained from one-photon fluorescence microscopy experiments. These findings indicate that probe **1** is suitable for imaging K^+ in living cells as a two-photon excited fluorescent K^+ sensor.

In summary, a water-soluble, BODIPY-based, two-photon excited fluorescence turn-on sensor (**1**) was developed for K^+ imaging in living cells. The sensor emits red-NIR fluorescence with an emission maximum of 644 nm upon binding K^+, exhibiting very high selectivity in detecting K^+ over other

DOI: 10.1021/acsami.5b04506
ACS Appl. Mater. Interfaces 2015, 7, 17565−17568

biologically important metal cations. The two-photon absorption cross-section of **1** was determined to be 500 GM at 940 nm, which is desirable for two-photon fluorescence microscopy cell and tissue imaging. The consistent results obtained from one- and two-photon cell imaging experiments demonstrate that **1** is a promising two-photon fluorescent probe for in vivo K^+ sensing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b04506.

Information on the synthesis and corresponding characterization data for compounds **1**−**6**, dissociation constant determination, cell culture for imaging, MALDI-HRMS spectra, and ^{1}H and ^{13}C spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: belfield@njit.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge support from the National Science Foundation (CBET-1517273 and CHE-0832622) and the U.S. National Academy of Sciences (PGA-P210877).

■ REFERENCES

(1) Shieh, C.-C.; Coghlan, M.; Sullivan, J. P.; Gopalakrishnan, M. Potassium Channels: Molecular Defects, Diseases, and Therapeutic Opportunities. *Pharmacol. Rev.* **2000**, *52*, 557−594.

(2) Wickenden, A. D. K+ Channels as Therapeutic Drug Targets. *Pharmacol. Ther.* **2002**, *94*, 157−182.

(3) Kofuji, P.; Newman, E. A. Potassium Buffering in the Central Nervous System. *Neuroscience* **2004**, *129*, 1043−1054.

(4) Bonnet, S.; Archer, S. L.; Allalunis-Turner, J.; Haromy, A.; Beaulieu, C.; Thompson, R.; Lee, C. T.; Lopaschuk, G. D.; Puttagunta, L.; Bonnet, S.; Harry, G.; Hashimoto, K.; Porter, C. J.; Andrade, M. A.; Thebaud, B.; Michelakis, E. D. A Mitochondria-K+ Channel Axis Is Suppressed in Cancer and Its Normalization Promotes Apoptosis and Inhibits Cancer Growth. *Cancer Cell* **2007**, *11*, 37−51.

(5) MacGregor, L. C.; Matschinsky, F. M. Altered Retinal Metabolism in Diabetes. II. Measurement of Sodium-Potassium Atpase and Total Sodium and Potassium in Individual Retinal Layers. *J. Biol. Chem.* **1986**, *261*, 4052−4058.

(6) Brennan, C. H.; Lewis, A.; Littleton, J. M. Membrane Receptors, Involved in up-Regulation of Calcium Channels in Bovine Adrenal Chromaffin Cells, Chronically Exposed to Ethanol. *Neuropharmacology* **1989**, *28*, 1303−1307.

(7) Kager, H.; Wadman, W. J.; Somjen, G. G. Simulated Seizures and Spreading Depression in a Neuron Model Incorporating Interstitial Space and Ion Concentrations. *J. Neurophysiol.* **2000**, *84*, 495−512.

(8) Gonçalves, A. R.; El Nahas, A. M. High Serum Potassium Levels after Using Losartan Can Reflect More Severe Renal Disease. *Diabetologia* **2011**, *54*, 2963−2964.

(9) Jezek, P.; Mahdi, F.; Garlid, K. D. Reconstitution of the Beef Heart and Rat Liver Mitochondrial K+/H+ (Na+/H+) Antiporter. Quantitation of K+ Transport with the Novel Fluorescent Probe, Pbfi. *J. Biol. Chem.* **1990**, *265*, 10522−10526.

(10) He, H.; Mortellaro, M. A.; Leiner, M. J. P.; Fraatz, R. J.; Tusa, J. K. A Fluorescent Sensor with High Selectivity and Sensitivity for Potassium in Water. *J. Am. Chem. Soc.* **2003**, *125*, 1468−1469.

(11) Padmawar, P.; Yao, X.; Bloch, O.; Manley, G. T.; Verkman, A. S. K+ Waves in Brain Cortex Visualized Using a Long-Wavelength K +-Sensing Fluorescent Indicator. *Nat. Methods* **2005**, *2*, 825−827.

(12) Hirata, T.; Terai, T.; Komatsu, T.; Hanaoka, K.; Nagano, T. Development of a Potassium Ion-Selective Fluorescent Sensor Based on 3-Styrylated Bodipy. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6090−6093.

(13) Zhou, X.; Su, F.; Tian, Y.; Youngbull, C.; Johnson, R. H.; Meldrum, D. R. A New Highly Selective Fluorescent K+ Sensor. *J. Am. Chem. Soc.* **2011**, *133*, 18530−18533.

(14) Sui, B.; Yue, X.; Tichy, M. G.; Liu, T.; Belfield, K. D. Improved Synthesis of the Triazacryptand (Tac) and Its Application in the Construction of a Fluorescent TAC-BODIPY Conjugate for K+ Sensing in Live Cells. *Eur. J. Org. Chem.* **2015**, *2015*, 1189−1192.

(15) Helmchen, F.; Denk, W. Deep Tissue Two-Photon Microscopy. *Nat. Methods* **2005**, *2*, 932−940.

(16) Bort, G.; Gallavardin, T.; Ogden, D.; Dalko, P. I. From One-Photon to Two-Photon Probes: "Caged" Compounds, Actuators, and Photoswitches. *Angew. Chem., Int. Ed.* **2013**, *52*, 4526−4537.

(17) Denk, W.; Strickler, J.; Webb, W. Two-Photon Laser Scanning Fluorescence Microscopy. *Science* **1990**, *248*, 73−76.

(18) Zipfel, W. R.; Williams, R. M.; Webb, W. W. Nonlinear Magic: Multiphoton Microscopy in the Biosciences. *Nat. Biotechnol.* **2003**, *21*, 1369−1377.

(19) Kim, H. M.; Jung, C.; Kim, B. R.; Jung, S.-Y.; Hong, J. H.; Ko, Y.-G.; Lee, K. J.; Cho, B. R. Environment-Sensitive Two-Photon Probe for Intracellular Free Magnesium Ions in Live Tissue. *Angew. Chem., Int. Ed.* **2007**, *46*, 3460−3463.

(20) Kim, H. M.; Kim, B. R.; An, M. J.; Hong, J. H.; Lee, K. J.; Cho, B. R. Two-Photon Fluorescent Probes for Long-Term Imaging of Calcium Waves in Live Tissue. *Chem. - Eur. J.* **2008**, *14*, 2075−2083.

(21) Kim, H. M.; Seo, M. S.; An, M. J.; Hong, J. H.; Tian, Y. S.; Choi, J. H.; Kwon, O.; Lee, K. J.; Cho, B. R. Two-Photon Fluorescent Probes for Intracellular Free Zinc Ions in Living Tissue. *Angew. Chem., Int. Ed.* **2008**, *47*, 5167−5170.

(22) Kim, M. K.; Lim, C. S.; Hong, J. T.; Han, J. H.; Jang, H.-Y.; Kim, H. M.; Cho, B. R. Sodium-Ion-Selective Two-Photon Fluorescent Probe for in Vivo Imaging. *Angew. Chem., Int. Ed.* **2010**, *49*, 364−367.

(23) Lim, C. S.; Han, J. H.; Kim, C. W.; Kang, M. Y.; Kang, D. W.; Cho, B. R. A Copper(I)-Ion Selective Two-Photon Fluorescent Probe for in Vivo Imaging. *Chem. Commun.* **2011**, *47*, 7146−7148.

(24) Buyukcakir, O.; Bozdemir, O. A.; Kolemen, S.; Erbas, S.; Akkaya, E. U. Tetrastyryl-Bodipy Dyes: Convenient Synthesis and Characterization of Elusive near Ir Fluorophores. *Org. Lett.* **2009**, *11*, 4644−4647.

(25) Bozdemir, O. A.; Guliyev, R.; Buyukcakir, O.; Selcuk, S.; Kolemen, S.; Gulseren, G.; Nalbantoglu, T.; Boyaci, H.; Akkaya, E. U. Selective Manipulation of Ict and Pet Processes in Styryl-Bodipy Derivatives: Applications in Molecular Logic and Fluorescence Sensing of Metal Ions. *J. Am. Chem. Soc.* **2010**, *132*, 8029−8036.

(26) Heathcote, R.; Howell, J. A. S.; Jennings, N.; Cartlidge, D.; Cobden, L.; Coles, S.; Hursthouse, M. Gold(I)-Isocyanide and Gold(I)-Carbene Complexes as Substrates for the Laser Decoration of Gold onto Ceramic Surfaces. *Dalton Trans.* **2007**, 1309−1315.

(27) Zheng, Q.; Xu, G.; Prasad, P. N. Conformationally Restricted Dipyrromethene Boron Difluoride (Bodipy) Dyes: Highly Fluorescent, Multicolored Probes for Cellular Imaging. *Chem. - Eur. J.* **2008**, *14*, 5812−5819.

(28) Didier, P.; Ulrich, G.; Mely, Y.; Ziessel, R. Improved Push-Pull-Push E-Bodipy Fluorophores for Two-Photon Cell-Imaging. *Org. Biomol. Chem.* **2009**, *7*, 3639−3642.

(29) Sheik-Bahae, M.; Said, A. A.; Wei, T. H.; Hagan, D. J.; Van Stryland, E. W. Sensitive Measurement of Optical Nonlinearities Using a Single Beam. *IEEE J. Quantum Electron.* **1990**, *26*, 760−769.

(30) Andersson, B.; Janson, V.; Behnam-Motlagh, P.; Henriksson, R.; Grankvist, K. Induction of Apoptosis by Intracellular Potassium Ion Depletion: Using the Fluorescent Dye Pbfi in a 96-Well Plate Method in Cultured Lung Cancer Cells. *Toxicol. In Vitro* **2006**, *20*, 986−994.

DOI: 10.1021/acsami.5b04506
ACS Appl. Mater. Interfaces 2015, 7, 17565−17568