JOC The Journal of Organic Chemistry

NOTE

pubs.acs.org/joc

Two-Photon Lysotrackers for in Vivo Imaging

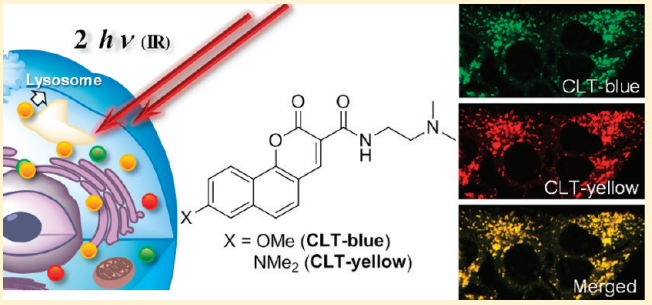
Ji Hoon Son,[†] Chang Su Lim,[†] Ji Hee Han,[†] Isravel Antony Danish,[†] Hwan Myung Kim,[‡] and Bong Rae Cho*[,†]

[†]Department of Chemistry, Korea University, 1-Anamdong, Seoul 136-701, Korea

[‡]Division of Energy Systems Research, Ajou University, Suwon 443-749, Korea

Supporting Information

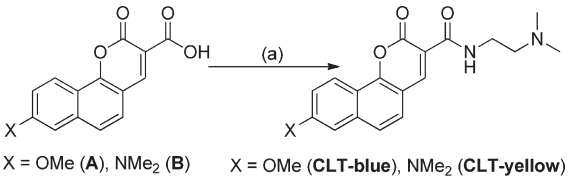
ABSTRACT: We report two-photon Lysotrackers (CLT-blue and CLT-yellow) that can be excited by 750−840 nm femtosecond laser pulses and emit at 470 and 550 nm, respectively. They can be easily loaded into cells and tissue slices for visualization of lysosomes in live cells and tissues for a long period of time through two-photon microscopy. When combined with appropriate two-photon probes for other biological targets, these novel probes would greatly facilitate the two-photon microscopy colocalization experiments.



Lysosomes are acidic organelles that constitute the terminal degrative compartments in mamalian cells.[1] They receive and degrade macromolecules through the phagocytic, endocytic, and autophagic pathways and play vital roles in various physiological processes.[1] To understand their roles in physiology, various lysosomal probes have been developed, some of them being commercially available.[2,3] However, use of these probes with one-photon microscopy (OPM) requires excitation with short-wavelength light (~350−550 nm) that limits their application in tissue imaging because of shallow penetration depth, photobleaching, and cellular autofluorescence. Overcoming these problems requires two-photon (TP) probes that can detect lysosomes in live cells and deep inside tissues by using two-photon microscopy (TPM). TPM, which uses two lower-energy photons for the excitation, is a new technique that can visualize deep inside intact tissues for an extended period of time.[4,5]

The most common method of confirming whether a biological activity occurs in lysosomes is using colocalization experiments in which cells are colabeled with fluorescent probes for the specific targets as well as for the lysosomes. To conduct such experiments in a two-photon mode, the TP probes for lysosomes and other targets must emit fluorescence in widely different wavelength regions. However, TP probes for lysosomes are rare.[6] Moreover, all of them emit TP excited fluorescence (TPEF) near 500 nm, which is too close to the emission bands of the existing TP probes for other targets to be useful.[7] We, therefore, have designed lysosomal TP probes that emit fluorescence in the short [CLT-blue (λ^{fl}_{max} = 470 nm)] and long wavelength regions [CLT-yellow (λ^{fl}_{max} = 550 nm)]. We have employed chromene as the fluorophore because it has shown a significant TP cross section.[8a,b] We also introduced different donors to tune the emission wavelengths and used the ethylenediamine moiety as the protonation site, with the expectation that the probes will accumulate in the acidic vesicles as the protonated form, thereby emiting strong TPEF in the lysosomes (Scheme 1).[6c] Herein, we report that both of these probes can detect lysosomes in live cells and intact tissues at depths of >100 μm by TPM.

Scheme 1. Synthesis of CLT-blue and CLT-yellow[a]



[a] (a) $H_2NCH_2CH_2NMe_2$, DCC, HOBt.

CLT-blue and CLT-yellow were prepared by the coupling of **A** and **B** with *N*,*N*-dimethylethylenediamine as shown in Scheme 1. The solubilities of CLT-blue and CLT-yellow in universal buffer (pH 7.0) as determined by the fluorescence method[9] were 3.0 and 2.5 μM, respectively, which affords concentrations that are sufficient to stain cells (Figure S2 of the Supporting Information).

In spectroscopic studies conducted in universal buffer (pH 7.0), the absorption maximum for CLT-blue appeared at 389 nm (ε = 22000 M^{-1} cm^{-1}) while that for CLT-yellow was well separated, by 60 nm, at 449 nm (ε = 19100 M^{-1} cm^{-1}). The fluorescence emission maxima (λ^{fl}_{max}) were even more widely separated from 477 nm (Φ = 0.88) for CLT-blue to 580 nm (Φ = 0.20) for CLT-yellow (Figure 1a and Table S1 of the

Received: July 21, 2011
Published: August 12, 2011

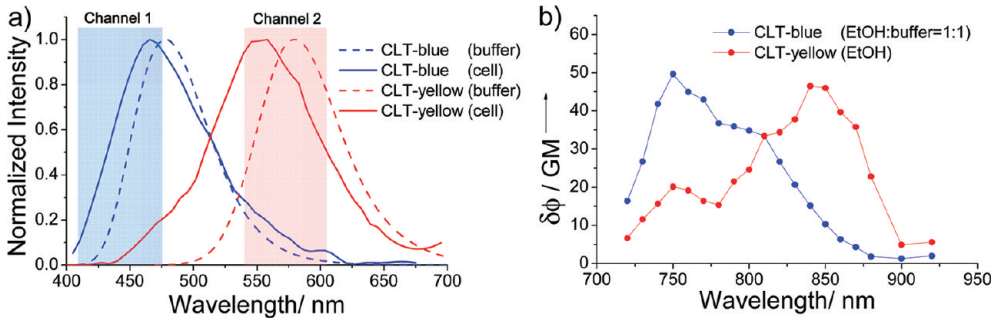


Figure 1. (a) Normalized one-photon (---) and two-photon fluorescence emission spectra (—) of CLT-blue (blue) and CLT-yellow (red) measured in universal buffer (pH 7.0) and HeLa cells, respectively. The excitation wavelengths for one- and two-photon processes were 389 and 750 nm for CLT-blue and 449 and 850 nm for CLT-yellow. (b) Two-photon action spectra of CLT-blue and CLT-yellow in an EtOH/buffer mixture (1/1) and EtOH, respectively. In all experiments, the probe concentration was 2 μM.

Table 1. Photophysical Data for Various Probes for Lysosomes

compd	λ_{max} [a]	λ^{fl}_{max} [b]	$\lambda^{(2)}_{max}$ [c]	$\Phi\delta_{max}$ [d]
CLT-blue[e]	389	471	750	50
CLT-yellow[f]	446	549	840	47
AL1[g]	364	496	780	92
LT1[h]	387	510	700	431
LTB	373[i]	422[i]	*j*	*j*
LTR	577[i]	599[i]	920[k]	8.5[k]

[a] λ_{max} of the one-photon absorption spectra in nanometers. [b] λ_{max} of the one-photon fluorescence spectra in nanometers. [c] λ_{max} of the two-photon absorption spectra in nanometers. [d] The two-photon action cross section in 10^{-50} cm^4/photon (GM), ±15%. [e] The solvent was an EtOH/buffer mixture (1/1). [f] The solvent was EtOH. [g] From ref 6c. [h] From ref 6a. [i] From ref 3. [j] The two-photon-excited fluorescence intensity was too weak to allow accurate measurement of the cross section, so this value was not determined. [k] The solvent was universal buffer (pH 7.0).

Supporting Information). The fluorescence spectra of CLT-blue and CLT-yellow show gradual bathochromic shifts with solvent polarity (E_T^N) in the following order: 1,4-dioxane < DMF < EtOH < universal buffer (Figure S1 and Table S1 of the Supporting Information). The shift is larger for CLT-yellow (43 nm vs 81 nm) presumably as a result of enhanced intramolecular charge transfer (ICT) by the stronger donor group. In HeLa cells, the emission maxima were blue-shifted from those in buffer, indicating that the probe environment in the cells is slightly more hydrophobic than the buffer (Figure 1a). Moreover, the shift was larger for CLT-yellow, providing additional support for the interpretation given above. Further, the emission maxima of CLT-blue (470 nm) and CLT-yellow (550 nm) in the cells are similar to those measured in an EtOH/buffer mixture (1/1) and EtOH, respectively, indicating that these solvents can adquately represent the polarity of the cellular environments.

The TP action cross section was determined by the fluorescence method as reported previously.[8c] The TP action spectra of CLT-blue and CLT-yellow in an EtOH/buffer mixture (1/1) and EtOH, which are good models for the intracellular environments (vide supra), indicated TP action cross section ($\Phi\delta_{max}$) values of 50 and 47 GM at 750 and 840 nm, respectively. The values are smaller than that of AL1 but much larger than those of LTR and LTB (Figure 1b and Table 1).[8] These values allowed us to obtain bright TPM images of the live cells and living tissues by TPM (vide infra).

To assess whether CLT-blue can detect lysosomes in live cells, HeLa cells were colabeled with CLT-blue and Lysotracker Red DND-99 (LTR), BODIPY-TR ceramide complexed to bovine serum albumin (BSA) (BTC), or Mitotracker Red FM (MTR), which are well-known one-photon fluorescent probes for the lysosome, Golgi apparatus, or mitochondria,[3] respectively. For this experiment, the detection windows of the probes were determined such that the emission intensities from the two probes under comparison should be almost the same (Figures S4 and S5 of the Supporting Information). The results show that the TPM image of CLT-blue overlapped well with the OPM image of the lysosome (Figure 2c), but not with those of Golgi apparatus and mitochondria (Figure S6 of the Supporting Information). The Pearson's colocalization coefficient, *A*, which describes the correlation of the intensity distribution between the channels,[10] was calculated by using Autoquant X2. The *A* value of CLT-blue with LTR was 0.89, a value much larger than the values of 0.17 and 0.035 obtained with MTR and BTC, respectively (Figure S6 and Table S2 of the Supporting Information). Experiments in NIH3T3 and A549 cells gave similar results (Figure S6 and Table S2 of the Supporting Information). These results establish that CLT-blue can serve as a TP probe for lysosomes in live cells.

We then evaluated the ability of CLT-yellow to detect lysosomes in live cells. The TPM images of the cells colabeled with CLT-yellow and Lysotracker blue (LTB) overlapped well with the OPM image of lysosomes, with *A* values of 0.87−0.91 (Table S2 of the Supporting Information), demonstrating the ability of CLT-yellow to detect lysosomes (Figure 2f).

We next sought to apply CLT-blue and CLT-yellow as TP probes for detecting lysosomes in a TP mode. The TPM images of the cells colabeled with CLT-blue and CLT-yellow showed almost perfect overlap with an *A* value of 0.92−0.94 (Figure 2i and Table S2 of the Supporting Information). The superior colocalization results between the two TPM images can be attributed to two factors. First, the two TPM images can be obtained simultaneously, whereas the TPM and OPM images must be obtained one after another; in the interval, the lysosomes can move. Second, the two TPM images compare the lysosomal distribution in the same $x-y$ plane, whereas OPM images reveal those in the entire depth of the cell. Thus, using two TP probes provides distinct advantages for colocalization experiments.

dx.doi.org/10.1021/jo201504h |*J. Org. Chem.* 2011, 76, 8113–8116

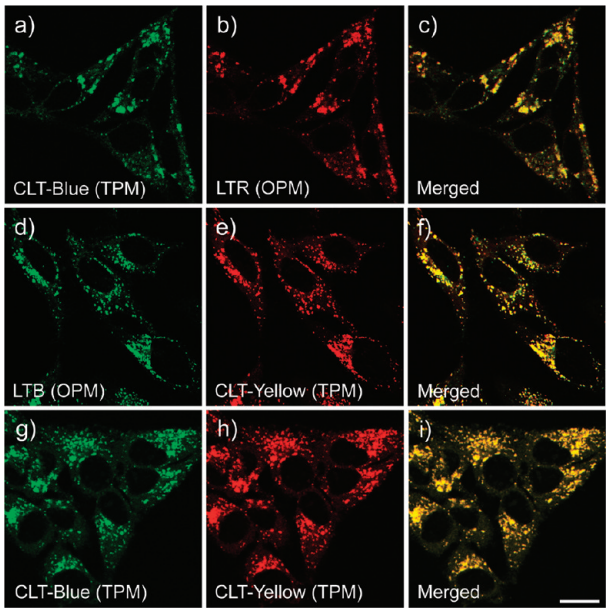


Figure 2. OPM, TPM, and merged images of HeLa cells colabeled with (a−c) CLT-blue (2 μM) and LTR (1 μM), (d−f) LTB (1 μM) and CLT-yellow (2 μM), (g−i) CLT-blue (2 μM) and CLT-yellow (2 μM), respectively. Except for panels g and h, the excitation wavelengths were 543 (LTR), 365 (LTB), 750 (CLT-blue), and 840 nm (CLT-yellow), the OP fluorescence was collected at 400−450 (LTB) and 580−700 nm (LTR), and TPEF was collected at 400−520 (CLT-blue) and 550−625 nm (CLT-yellow). (g and h) For colocalization between CLT-blue and CLT-yellow, the probes were excited at 750 nm and the TPEF was recorded at 400−475 (CLT-blue) and 550−625 nm (CLT-yellow), respectively. Areas of colocalization appear in orange-red. The scale bar is 20 μm. Cells shown are representative images from replicate experiments (n = 5).

CLT-blue and CLT-yellow have the additional benefits of negligible cytotoxicity as measured by using a CCK-8 kit (Figure S8 of the Supporting Information), pH-independent fluorescence intensities in the biologically relevant pH range (Figure S3 of the Supporting Information) and high photostabilities are revealed by the negligible changes in the TPEF intensities in the probe-labeled HeLa cells over 60 min (Figure S7 of the Supporting Information).

We further investigated the utility of CLT-blue and CLT-yellow in tissue imaging. The bright field image of a section of fresh rat hippocampal slice from the postnatal 2-week-old rat incubated with 20 μM CLT-blue and CLT-yellow for 30 min at 37 °C reveals the CA1 and CA3 regions as well as the dentate gyrus (DG) (Figure 3a). The dual-channel TPM images and the merged image show the lysosome distributions in the same region at a depth of 120 μm (Figure 3a and Figure S9 of the Supporting Information). The TPM images obtained at depths of 80−170 μm reveal that the lysosomes are almost evenly distributed throughout the entire depth (Figure S10 of the Supporting Information). Moreover, the image taken at a higher magnification clearly resolved the lysosome distributions in the pyramidal neuron layer of the CA3 region (Figure 3b−d). Further, the TPM images of the tissue slices labeled with CLT-blue and CLT-yellow overlapped almost completely with an A value of 0.96 (Table S2 of the Supporting Information). Hence, CLT-blue and CLT-yellow are clearly capable of visualizing the lysosomes at depths of 80−170 μm in live tissues by TPM.

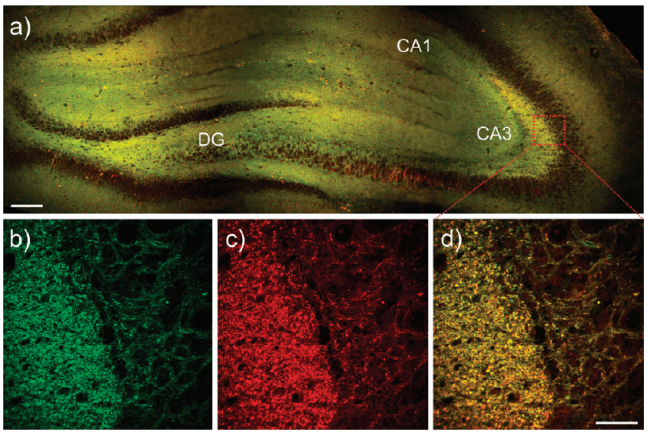


Figure 3. Dual-channel TPM images of a rat hippocampal slice colabeled with CLT-blue (20 μM) and CLT-yellow (20 μM). The TPEF was collected at 400−475 (CLT-blue, Ch 1) and 550−625 nm (CLT-yellow, Ch 2). (a) TPM images of the CA1−CA3 regions and the dentate gyrus (DG) with 10× magnification at a depth of approximately 120 μm in the two channels and then merged. (b−d) TPM images of CA3 regions (red box in panel a) collected at (b) Ch 1 and (c) Ch 2 at a depth of ∼100 μm at 100× magnification. (d) Merged image of panels b and c. The excitation wavelength was 750 nm. Scale bars are 30 (b−d) and 225 μm (a).

It is worth noting that CLT-blue and CLT-yellow are more useful than other lysosmal TP probes for colocalization experiments in live cells and living tissues. Although the $\Phi\delta_{max}$ values of AL1 and LT1 are much larger than those of CLT-blue and CLT-yellow, their emission maxima are at ∼500 nm, which is too close to those of the TP probes for other targets to be useful for colocalization.[6,7] In addition, the utitlity of LT1 in tissue imaging has not been established. On the other hand, CLT-blue and CLT-yellow exhibit sufficient water solubility, good cell permeability, significant $\Phi\delta_{max}$ values to obtain bright TPM images, and high photostability. Most importantly, their emission maxima are widely separated from those of existing TP probes to make them useful for the colocalization experiment with TP probes for other targets.

To conclude, we have developed TP Lysotrackers (CLT-blue and CLT-yellow) that can be excited by 750−840 nm femtosecond laser pulses and emit TPEF at 400−475 and 550−625 nm, respectively. They can be easily loaded into cells and tissues and used to visualize the lysosomes in live cells and tissues for a long period of time by TPM with minimal interference by pH or cytotoxicity. When combined with appropriate TP probes for other biological targets, these novel probes would greatly facilitate the TPM colocalization experiments.

EXPERIMENTAL SECTION

Synthesis. 8-Methoxy-2-oxo-2*H*-benzo[*h*]chromene-3-carboxylic acid (**A**) and the 8-dimethylamino derivative (**B**) were available from previous studies.[8a,b] Syntheses of CLT-blue and CLT-yellow are described below.

CLT-blue. A mixture of **A** (0.50 g, 1.9 mmol), DCC (0.42 g, 2.0 mmol), and HOBt (0.30 g, 2.2 mmol) in CH_2Cl_2 (20 mL) was added slowly to *N*,*N*-dimethylethane-1,2-diamine (0.18 g, 2.0 mmol), and the mixtures were refluxed for 4 h. The solvent was evaporated, and the crude product was purified by column chromatography using a $CHCl_3$/MeOH mixture (4/1) as the eluent: yield 0.41 g (65%); mp 179−180 °C; IR (KBr)

dx.doi.org/10.1021/jo201504h |*J. Org. Chem.* 2011, 76, 8113–8116

1750, 1680 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.96 (s, 1H), 8.46 (d, 1H, J = 9.2 Hz), 7.63 (d, 1H, J = 8.8 Hz), 7.56 (d, 1H, J = 8.8 Hz), 7.32 (dd, 1H, J = 2.4, 9.2 Hz), 7.19 (d, 1H, J = 2.4 Hz), 3.97 (s, 3H), 3.60 (t, 2H, J = 6.3 Hz), 2.56 (t, 2H, J = 6.3 Hz), 2.31 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.3, 162.3, 161.1, 153.0, 149.0, 138.3, 125.5, 124.5, 124.4, 120.1, 117.6, 116.5, 113.1, 106.9, 58.2, 55.8, 45.6, 37.9; HRMS (FAB^+) m/z calcd for $[C_{19}H_{20}N_2O_4 + H^+]$ 341.1501, found 341.1503.

CLT-yellow. Synthesized from **B** by the same procedure described for CLT-blue: yield 64%; mp 230−231 °C; IR (KBr) 1740, 1675 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.86 (s, 1H), 8.35 (d, 1H, J = 9.4 Hz), 7.45 (d, 1H, J = 9.1 Hz), 7.40 (d, 1H, J = 9.1 Hz), 7.18 (dd, 1H, J = 2.2, 9.4 Hz), 6.82 (d, 1H, J = 2.2 Hz), 3.60 (t, 2H, J = 6.3 Hz), 3.14 (s, 6H), 2.57 (t, 2H, J = 6.3 Hz), 2.32 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.8, 162.3, 153.8, 151.2, 149.0, 138.6, 125.1, 124.5, 124.9, 116.0, 114.3, 114.1, 111.7, 105.8, 58.3, 45.6, 40.5, 37.9; HRMS (FAB^+) m/z calcd for $[C_{19}H_{20}N_2O_4 + H^+]$ 354.1818, found 354.1816.

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra, photophysical studies, TPM imaging of the cells and tissue slices, and colocalization experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: chobr@korea.ac.kr.

Author Contributions
J.H.S. and C.S.L. contributed equally to this work.

■ ACKNOWLEDGMENT

This work was supported by National Research Foundation (NRF) grants funded by the Korean Government (2011-0020477, 2011-0002316, and 2011-0004321), the Priority Research Centers Program through the NRF funded by the Ministry of Education, Science and Technology (2011-0018396 and 2010-0028294), and an Ajou university research fellowship of 2010. C.S.L. and J.H.H. were supported by a BK21 scholarship.

■ REFERENCES

(1) (a) Saftig, P.; Klumperman, J. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 623. (b) Luzio, J. P.; Pryor, P. R.; Bright, N. A. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 622.

(2) Anderson, R. G. W.; Orci, L. *J. Cell Biol.* **1988**, *106*, 539.

(3) Haugland, R. P., Ed. *A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed.; Molecular Probes: Eugene, OR, 2005.

(4) (a) Helmchen, F.; Denk, W. *Nat. Methods* **2005**, *2*, 932. (b) Zipfel, W. R.; Williams, R. M.; Webb, W. W. *Nat. Biotechnol.* **2003**, *2*, 1369.

(5) (a) Kim, H. M.; Cho, B. R. *Acc. Chem. Res.* **2009**, *42*, 863. (b) Kim, H. M.; Cho, B. R. *Chem.—Asian J.* **2011**, *6*, 58.

(6) (a) Wang, X.; Nguyen, D. M.; Yanez, C. O.; Rodriguez, L.; Ahn, H.-Y.; Bonder, M. V.; Belfield, K. D. *J. Am. Chem. Soc.* **2010**, *132*, 12237. (b) Yao, S.; Ahn, H.-Y.; Wang, X.; Fu, J.; Van Stryland, E. W.; Hagan, D. J.; Belfield, K. D. *J. Org. Chem.* **2010**, *75*, 3965. (c) Kim, H. M.; An, M. J.; Hong, J. H.; Jeong, B. H.; Lee, K. J.; Kwon, O.; Cho, B. R. *Angew. Chem., Int. Ed.* **2008**, *47*, 2231.

(7) (a) Sumalekshmy, S.; Fahrni, C. J. *Chem. Mater.* **2011**, *23*, 483. (b) Kim, H. M.; Cho, B. R. *Acc. Chem. Res.* **2009**, *42*, 863. (c) Kim, H. M.; Cho, B. R. *Chem.—Asian J.* **2011**, *6*, 58.

(8) (a) Kim, H. M.; Fang, X. Z.; Yang, P. R.; Yi, J.-S.; Ko, Y.-G.; Piao, M. J.; Chung, Y. D.; Park, Y. W.; Jeon, S.-J.; Cho, B. R. *Tetrahedron Lett.* **2007**, *48*, 2791. (b) Kim, H. M.; Yang, P. R.; Jung, C.; Kim, B. R.; Jung, S.-Y.; Ko, Y.-G.; Cho, B. R. *J. Org. Chem.* **2007**, *72*, 2088. (c) Lee, S. K.; Yang, W. J.; Choi, J. J.; Kim, C. H.; Jeon, S.-J.; Cho, B. R. *Org. Lett.* **2005**, *7*, 323.

(9) 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. *Angew. Chem., Int. Ed.* **2008**, *47*, 5167.

(10) Adler, J.; Parmryd, I. *Cytometry, Part A* **2010**, *77A*, 733.