Anal. Chem. **2006,** *78,* 8175−8182

Cell-Based Indicator to Visualize Picomolar Dynamics of Nitric Oxide Release from Living Cells

Moritoshi Sato,[†,‡] Takahiro Nakajima,[†] Mariko Goto,[†] and Yoshio Umezawa*[,†]

Department of Chemistry, School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan

We report a novel cell-based indicator that is able to visualize picomolar dynamics of nitric oxide release from living cells. Cells from a pig kidney-derived cell line (PK15) endogenously express soluble guanylate cyclase (sGC), which is a receptor protein for the selective recognition of NO. Binding of NO by sGC causes the amplified generation of guanosine 3′,5′-cyclic monophosphate (cGMP). To make the PK15 cells into NO indicators, the cells are transfected with a plasmid vector encoding a fluorescent indicator for cGMP and fluorescence resonance energy transfer is recorded at 480 ± 15 and 535 ± 12.5 nm upon excitation of the cells at 440 ± 10 nm. The cell-based indicator exhibits exceptional sensitivity (detection limit of 20 pM), selectivity, reversibility, and reproducibility. The outstanding sensitivity of the present indicator has led us to uncover an oscillatory release of picomolar concentrations of NO from hippocampal neurons. We present evidence that Ca^{2+} oscillations in hippocampal neurons underlie the oscillatory NO release from the neurons during neurotransmission. We also have succeeded in visualizing the extent of diffusing NO from single vascular endothelial cells. The present cell-based indicator provides a powerful tool to uncover picomolar dynamics of NO that regulates a wide range of cell functions in biological systems.

Nitric oxide is a small uncharged free radical that plays a key role in many biological processes.[1−3] The physicochemical property of NO allows it to act as a diffusible intercellular messenger like paracrine factors including cytokines and neurotransmitters.[1] It is thus of essential importance to analyze the spatiotemporal dynamics of NO release from the cell for the understanding of biological processes of NO. However, convincing methods are not available for the detection of NO release that provide the spatiotemporal information. If indicators become available for the detection of NO release from living cells, such a spatiotemporal dynamics of NO release may be visualized. Here we developed a novel cell-based indicator for NO with fluorescence readout.

The present cell-based indicator is in contrast to the existing fluorescent molecular indicators for NO, such as the genetically encoded indicator (NOA)[4] and organic indicators including diaminofluoresceins (DAFs),[5] diaminocyanines (DACs),[6] and a copper-based fluorescent indicator (CuFL).[7] These molecular indicators are introduced in NO-releasing cells and provide information inside the cells. On the other hand, the present cell-based indicator is placed outside the NO-releasing cell and thereby detects the NO release from the cells. We further show that the cell-based indicator exhibits exceptional sensitivity to picomolar concentrations of NO and is superior in the selectivity, reversibility, reproducibility, and experimental feasibility. The present cell-based indicator thus revealed picomolar dynamics of NO release from vascular endothelial cells and from hippocampal neurons, respectively.

EXPERIMENTAL SECTION

Materials. We purchased *N,N*′-bis(carboxymethyl)-*N,N*′-dinitroso-*p*-phenylenediamine disodium salt (caged NO) and p^3-[1-(2-nitrophenyl)ethyl]adenosine 5′-triphosphate trisodium salt (caged ATP) from Dojindo Laboratories (Kumamoto, Japan). *N*ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), *N*ω-nitro-D-arginine methyl ester hydrochloride (D-NAME), 4*H*-8-bromo-1,2,4-oxadiazolo[3,4-*d*]benz[b][1,4]oxazin-1-one (NS 2028), 3-isobutyl-1-methylxanthine (IBMX), D(−)-2-amino-5-phosphonopentanoic acid (APV) were purchased from Sigma Chemical Co. (St. Louis, MO). PK15 cells and bovine pulmonary artery endothelial cells were obtained from Japanese Collection of Research Bioresources (Osaka, Japan).

Development of Piccell. PK15 cells were transfected with a plasmid vector encoding the genetically encoded fluorescent indicator for guanosine 3′,5′-cyclic monophosphate (cGMP) using LipofectAMINE 2000 reagent. Transformants permanently expressed with the cGMP indicator were selected using 0.8 mg/mL of geneticin. A fluorescent clone, named Piccell, was used for experiments.

* To whom correspondence should be addressed: (phone) +81-3-5841-4351; (fax) +81-3-5841-8349; (e-mail) umezawa@chem.s.u-tokyo.ac.jp.

† The University of Tokyo.

‡ PRESTO, Japan Science and Technology Agency.

(1) Ignarro, L. J., Ed. *Nitric oxide: biology and pathobiology*; Academic Press: New York, 2000.

(2) Zhang, J.; Snyder, S. H. *Annu. Rev. Pharmacol. Toxicol.* **1995**, *35*, 2113−2133.

(3) Dudzinski, D. M.; Igarashi, J.; Greif, D.; Michel, T. *Annu. Rev. Pharmacol. Toxicol.* **2006**, *46*, 235−276.

(4) Sato, M.; Hida, N.; Umezawa, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 14515−14520.

(5) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446−2453.

(6) Sasaki, E.; Kojima, H.; Nishimatsu, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 3684−3685.

(7) Lim, M. H.; Xu, D.; Lippard, S. J. *Nat. Chem. Biol.* **2006**, *2*, 375−380.

10.1021/ac061791b CCC: $33.50
Published on Web 11/09/2006

Cell Culture. Piccell was cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids (Invitrogen, Carlsbad, CA), and 0.8 mg/mL geneticin at 37 °C in 5% CO_2/95% air. Primary hippocampal neurons were prepared from Wistar rat embryos (embryonic day 17) and cultured in Neurobasal medium supplemented with 2% B27 supplement and 0.5 mM glutamine at 37 °C in 5% CO_2/95% air. Vascular endothelial cells were cultured in Eagle's minimal essential medium supplemented with 20% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acids at 37 °C in 5% CO_2/95% air.

Preparation of NO and CO Solutions. To prepare saturated NO solution (2 mM), PBS was bubbled with argon gas for 1 h and then bubbled with NO gas for 1 h. Solutions containing various concentrations of NO were prepared by diluting the saturated NO solution with the deaerated PBS. CO-saturated solution (1 mM) was prepared by bubbling the deaerated PBS with CO gas for 1 h. Solutions containing various concentrations of CO were prepared by diluting the saturated CO solution.

Imaging of Cells. The culture medium was replaced with a Hank's balanced salt solution supplemented with 20 mM HEPES, pH 7.4. The cells were imaged at 25 °C on a Carl Zeiss Axiovert 200 microscope with a cooled CCD camera CoolSNAP HQ (Roper Scientific Inc, Tucson, AZ), controlled by MetaFluor (Universal Imaging, West Chester, PA). Upon excitation of cyan fluorescent protein (CFP) at 440 ± 10 nm, fluorescence images were obtained through a 480 ± 15 nm filter for CFP and 535 ± 12.5 nm filter for yellow fluorescent protein (YFP) with a 40× oil immersion objective (Carl Zeiss, Jena, Germany). All filters and dichroic mirror (455DRLP) were obtained from Omega Optical (Brattleboro, VT).

Uncaging of Caged Compounds. The caged NO and caged ATP were transiently uncaged by photolysis at 365 ± 12 nm from a xenon lamp to stimulate cells with NO and ATP, respectively. The local uncaging of the caged NO at a diameter of 20 μm was achieved by inserting a pinhole, the diameter of which is 100 μm, at the field diaphragm of the fluorescence microscope. After the uncaging of the caged NO or caged ATP, we observed CFP/YFP fluorescence resonance energy transfer (FRET) in Piccell at 480 ± 15 and 535 ± 12.5 nm upon excitation at 440 ± 10 nm. Because the caged NO and caged ATP are water-soluble, they may be cell-impermeable and confined in the extracellular space.

RESULTS

Construction of the Present Cell-Based Indicator for NO. Soluble guanylate cyclase (sGC) is a receptor protein for the selective recognition of NO in the cell[8] (Figure 1a). sGC consists of α- and β-subunits and a heme group, to which NO binds. After binding of NO to the heme group, the cyclase activity of sGC is stimulated up to 400-fold, resulting in the generation of cGMP in the cell.[9] Many cell types including a pig kidney-derived cell line (PK15) endogenously express the α- and β-subunits of sGC (Figure 1b). To develop a cell-based indicator for NO, we permanently expressed, in the PK15 cell, a genetically encoded molecular indicator for cGMP, which we have previously reported[10] (Figure 1a and c). The cGMP indicator having a donor (CFP) and acceptor (YFP) for FRET emits cGMP-dependent FRET signals. The PK15 cell expressing cGMP indicator thus emits FRET signals in response to NO through the generated cGMP (Figure 1a). As to the sensitivity of this cell-based indicator, because a single NO molecule stimulates the generation of cGMP at the rate of ~6000 molecules/min, it provokes FRET signals from a large number of the cGMP indicators in the cell-based indicator. This provides a molecular basis for the outstanding sensitivity of the present cell-based indicator. We confirmed that all the individual cell-based indicators emit the identical FRET signal in response to an NO stimulation (Figure 1d). Each independent and identical response of the individual cell-based indicators reminds us of each pixel of CCD cameras. We named this cell-based NO indicator "Piccell".

Sensitivity, Selectivity, Reversibility, and Reproducibility of Piccell. When we added 100 pM NO to Piccell, an emission ratio of CFP to YFP, which is a measure for FRET in the expressed cGMP indicator, immediately decreased and then recovered up to the initial level in 150 s (Figure 2a). The recovery of the decreased emission ratio disappeared when Piccell was pretreated with an inhibitor of phosphodiesterases that hydrolyze cGMP, 1 mM IBMX (Figure 2a). The NO-induced transient change in the CFP/YFP emission ratio, however, was completely blocked by pretreating Piccell with an inhibitor of sGC, 100 μM NS 2028 (Figure 2a). The result indicates that sGC generates cGMP in Piccell upon NO stimulation, and cGMP is hydrolyzed by endogenous phosphodiesterases. The expressed fluorescent indicator for cGMP detects the NO-induced change in the cGMP concentrations in Piccell.

To examine dose−response relationships, we added various concentrations of NO to Piccell. Figure 2b shows the averaged peak response of Piccell at each NO concentration. The result indicates that Piccell detects picomolar concentrations of NO (detection limit of 20 pM) (Figure 2b). We also examined the selectivity of Piccell. Carbon monoxide, atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and L-glutamate are, respectively, candidates for possible interfering factors. This is because CO is a weak activator for sGC: ANP and CNP activate their transmembrane receptors that generate cGMP: L-glutamate stimulates the production of adenosine 3′,5′-cyclic monophosphate (cAMP), and the cGMP indicator has a weak affinity with cAMP.[10] However, Piccell exhibited no significant responses to these factors up to 100 μM (Figure 2b). Piccell was thus found to be highly selective to NO.

We confirmed the reversibility and reproducibility of Piccell using caged NO, *N,N*′-bis(carboxymethyl)-*N,N*′-dinitroso-*p*-phenylenediamine.[11] UV irradiation photolyzes the caged NO and generates NO during the irradiation. Piccell was incubated with the caged NO and irradiated at 365 ± 12 nm for 1 s. We monitored the CFP/YFP emission ratio of Piccell before and after the transient NO release. Single UV irradiation induced a transient sharp response of Piccell (Figure 2c). Repeated UV irradiations

(8) Koesling, D.; Russwurm, M.; Mergia, E.; Mullershausen, F.; Friebe, A. *Neurochem. Int.* **2004**, *45*, 813−819.
(9) Katsuki, S.; Arnold, W.; Mittal, C.; Murad, F. *J. Cycl. Nucleotide Res.* **1977**, *3*, 23−35.
(10) Sato, M.; Hida, N.; Ozawa, T.; Umezawa, Y. *Anal. Chem.* **2000**, *72*, 5918−5924.
(11) Namiki, S.; Arai, T.; Fujimori, K. *J. Am. Chem. Soc.* **1997**, *119*, 3840−3841.

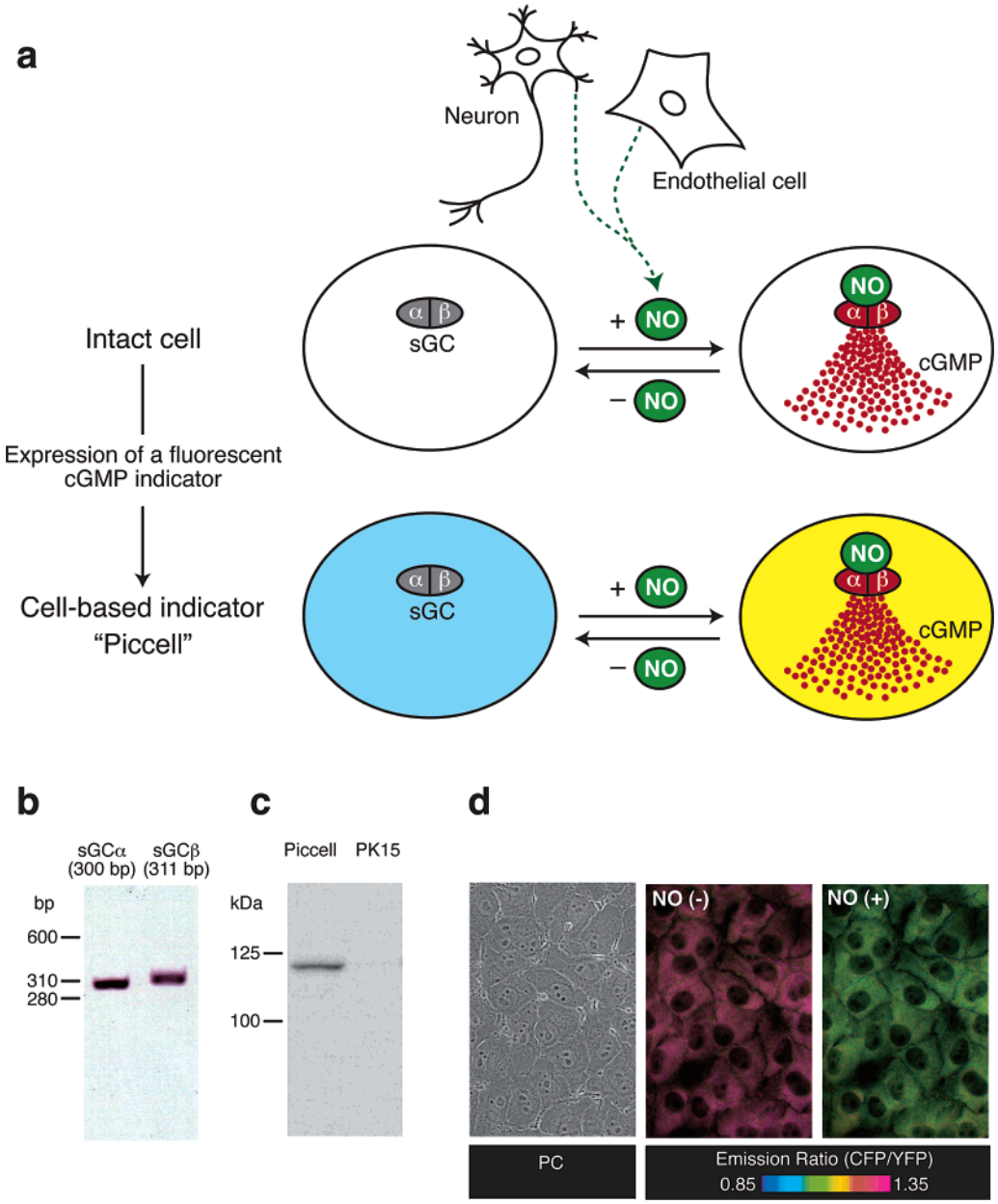

Figure 1. Cell-based fluorescent indicator for NO. (a) Development of the cell-based NO indicator "Piccell" that visualizes the NO release from living cells, such as neurons and vascular endothelial cells. sGC binds to NO and generates cGMP at the rate of ~6000 molecules/min. For the construction of Piccell, a fluorescent indicator for cGMP was expressed in the cell, as shown in cyan. The cGMP indicator emits a FRET signal in response to the NO-induced cGMP generation in Piccell, as shown in yellow. In Piccell, a single NO molecule triggers a large number of the cGMP indicators to emit FRET signals. This is why Piccell allows high-sensitivity detecion of NO. (b) RT-PCR analysis shows that PK15 cells endogenously express the α- and β-subunits of sGC. (c) Permanent expression of the cGMP indicator was examined by immunoblot analysis using anti-GFP antibody. A single band corresponding to the cGMP indicator was observed. (d) A phase contrast image of Piccell (left) and pseudocolor image of the CFP/YFP emission ratio of Piccell in the absence (center) or presence (right) of 10 nM NO.

every 5 min induced repeated sharp responses of Piccell (Figure 2c and d). The result confirms that Piccell reversibly detects NO and has enough reproducibility. The reversible response of Piccell is caused by the reversible binding of NO to sGC and phosphodiesterases that immediately hydrolyze cGMP after the removal of NO by its oxidation, volatilization, or both. Taken together, Piccell is an indicator having exceptional sensitivity to picomolar concentrations of NO, high selectivity, reversibility, and reproducibility.

Imaging Picomolar Dynamics of NO Release from Vascular Endothelial Cells. We cocultured the cell-based indicator Piccell with vascular endothelial cells (Figure 3a). Piccell visualizes the NO release from the endothelial cell adjacent to the Piccell (Figure 3a). Endothelial cells endogenously express G-protein-coupled purinergic receptors.[12] When the endothelial cell was stimulated with 100 nM adenosine 5′-triphosphate (ATP), Piccell visualized an ATP-induced transient NO release from the endothelial cell (Figure 3b). The peak response of Piccell indicates the release of 40 pM NO from the endothelial cell. Upon subsequent stimulation with 1 μM ATP, the Piccell visualized the release of

(12) Guns, P. J.; Korda, A.; Crauwels, H. M.; Assche, T. V.; Robaye, B.; Boeynaems, J. M.; Bult, H. *Br. J. Pharmacol.* **2005**, *146*, 288−295.

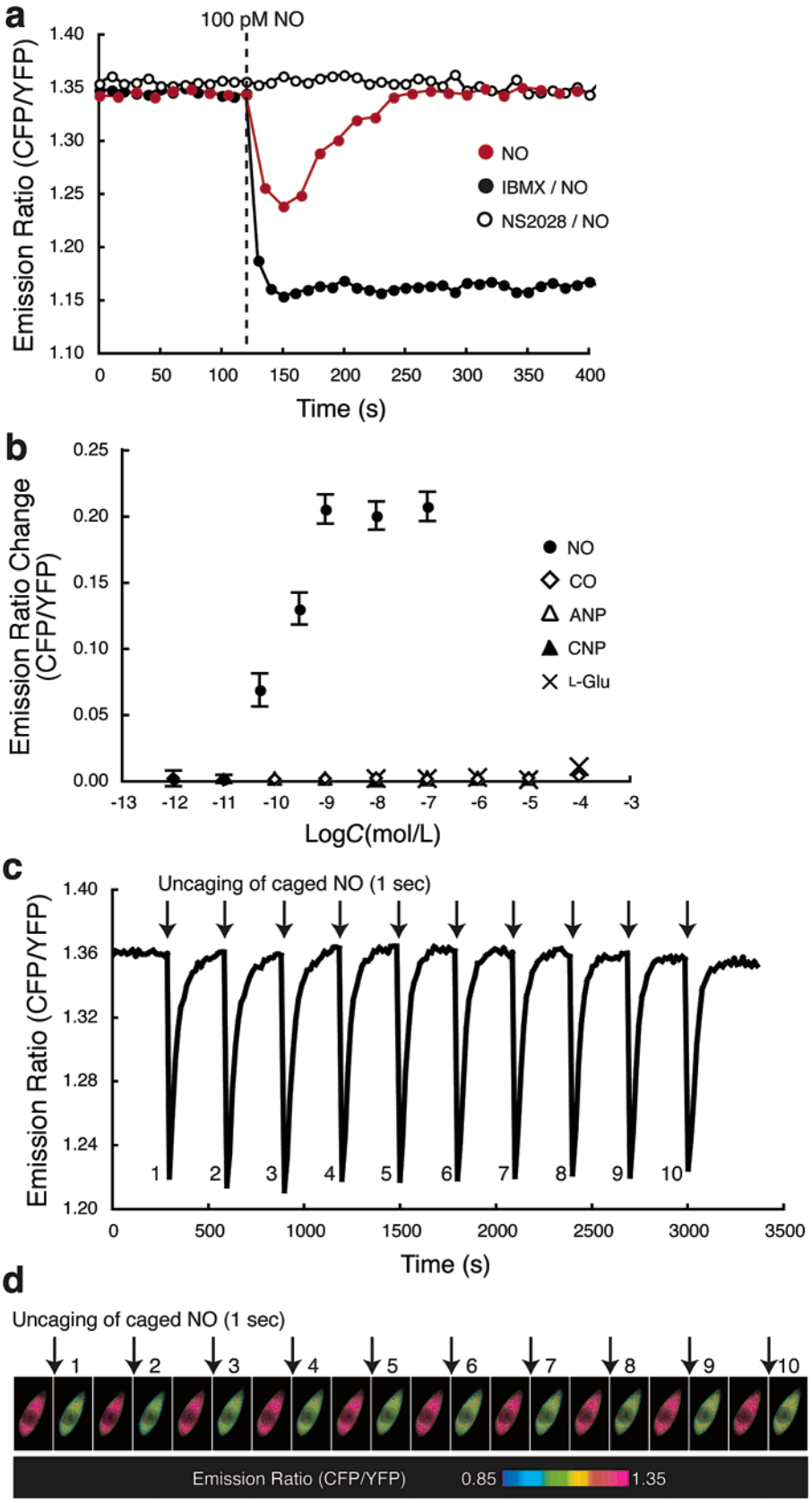

Figure 2. Piccell detects picomolar concentrations of NO. (a) Response of Piccell for 100 pM NO (red closed circle). Responses of Piccell are also shown for 100 pM NO in the presence of 1 mM IBMX (black closed circle) or 100 μM NS2028 (black open circle). (b) Dose−response relationships of Piccell for NO, CO, ANP, CNP, and L-glutamate. (c) Every time NO was generated for 1 s by photolysis of the caged NO, Piccell exhibited a sharp FRET response. The uncaging of the caged NO was repeated consecutive 10 times. The result confirms the reversibility and reproducibility of Piccell. (d) Pseudocolor images of the CFP/YFP emission ratio of Piccell before and after repeated uncaging of the caged NO in (c). The numbers 1−10 correspond to the repeated uncaging of the caged NO in (c).

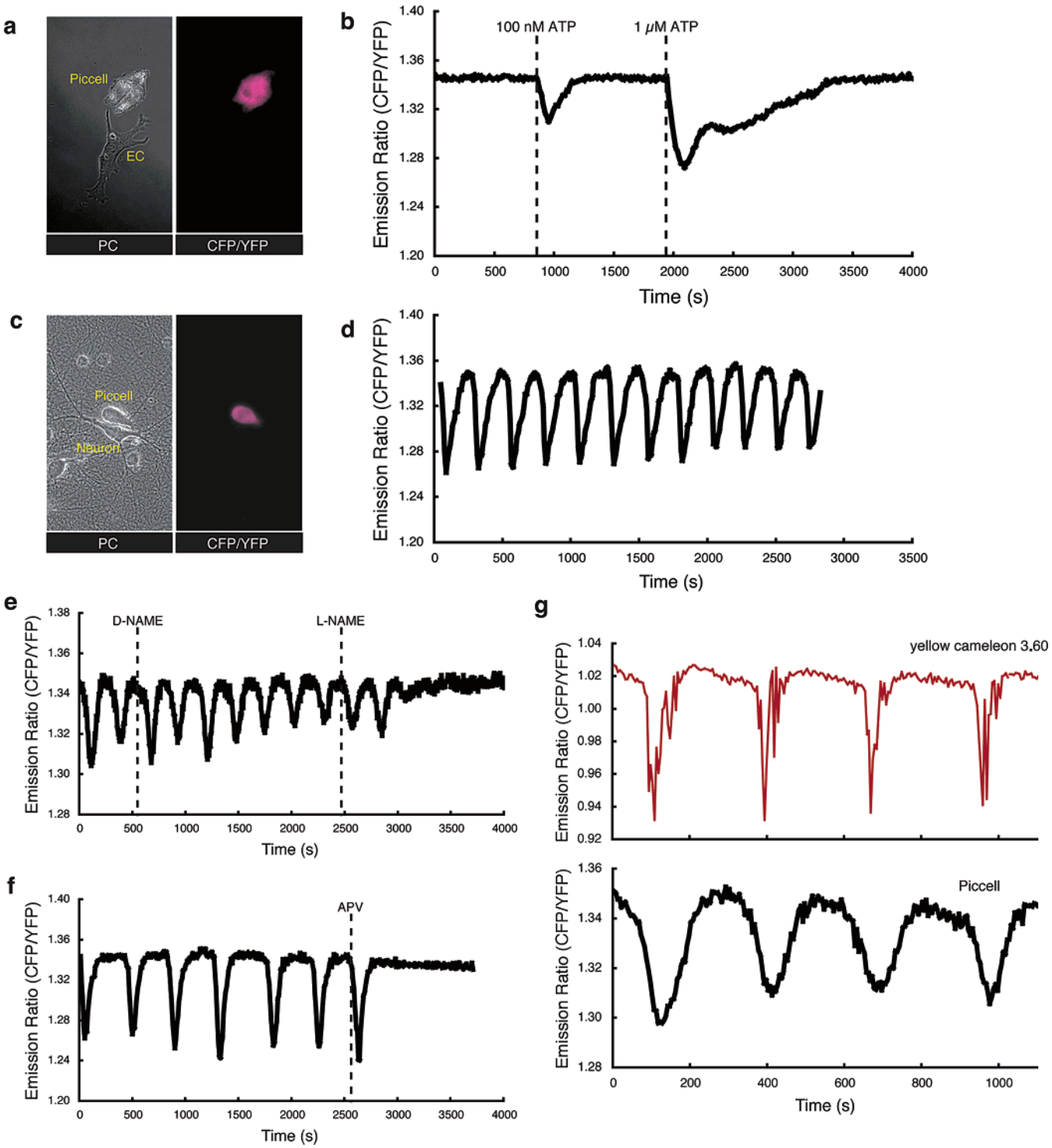

Figure 3. Visualization of the NO release from living cells with Picell. (a) A phase contrast image (left) and pseudocolor image of the CFP/YFP emission ratio (right) of a cocultured vascular endothelial cell and Piccell. (b) Time course of the FRET response of Piccell for 100 nM and subsequent 1 μM ATP stimulations, when Piccell was cocultured with the endothelial cell. (c) A phase contrast image (left) and pseudocolor image of the CFP/YFP emission ratio (right) of cocultured hippocampal neurons and Piccell. (d) Time course of the FRET response of Piccell cocultured with hippocampal neurons. (e) The oscillatory FRET response of Piccell cocultured with neurons is blocked with L-NAME but not D-NAME. (f) The oscillatory FRET response of Piccell cocultured with neurons is blocked with 100 μM APV. (g) Simultaneous analysis of neuronal NO release and intracellular Ca^{2+} concentrations in hippocampal neurons. Time course of the FRET response of yellow cameleon 3.60 in the hippocampal neuron (upper, red line) and that of Piccell (lower, black line). The oscillatory FRET response of Piccell synchronizes with that of yellow cameleon 3.60.

100 pM NO from the endothelial cell (Figure 3b). Piccell thus visualizes the picomolar dynamics of NO release from living cells.

Imaging Picomolar Dynamics of NO Release from Neurons. NO is a key factor that regulates neuronal functions such as neurotransmission, learning, and memory.[13] However, it has remained unclear to what extent and how NO is generated and

(13) Nelson, R. J.; Kriegsfeld, L. J.; Dawson, V. L.; Dawson, T. M. *Front. Neuroendocrinol.* **1997**, *18*, 463−491.

released from neurons. We next cocultured Piccell with hippocampal neurons to visualize the picomolar dynamics of neuronal NO (Figure 3c). Interestingly, Piccell cocultured with neurons exhibited very different FRET responses compared to the case of its coculture with endothelial cells. Even in the absence of additional stimuli, the CFP/YFP emission ratio of Piccell exhibited repeated cycles of its decrease and subsequent increase (Figure 3d). The oscillatory FRET response of Piccell disappeared by treating with an inhibitor of NO synthase, 100 μM L-NAME (Figure 3e). The 100 μM D-NAME, an inactive isomer of NAME, had no significant effect on the oscillatory FRET response of Piccell cocultured with neurons (Figure 3e). The result reveals that hippocampal neurons are releasing picomolar concentrations of NO spontaneously and periodically, that is NO oscillations. The averaged peak response of Piccell indicates the periodic release of 100 pM NO from neurons.

We wondered how neurons periodically release picomolar concentrations of NO. We examined an effect of an antagonist of glutamate receptor isoforms known as NMDA receptors[14] on the NO oscillations. When we added the antagonist of NMDA receptors, APV, the oscillatory NO release from neurons was completely blocked (Figure 3f). Tetrodotoxin, which blocks action potentials by inhibiting voltage-gated Na^+ channels, also blocked the oscillatory release of NO from neurons (Supporting Information Figure 1). The result indicates that the oscillatory release of picomolar concentrations of neuronal NO is under the control of NMDA receptors activated by spontaneous neurotransmission between neurons.

We further show that Ca^{2+} oscillations in hippocampal neurons underlie the oscillatory NO release from the neurons during spontaneous neurotransmission. Because NMDA receptors are Ca^{2+}-permeable channels, the receptors allow Ca^{2+} influx into neurons upon neurotransmission.[15] The influxed Ca^{2+} in neurons is believed to generate NO through the activation of neuronal NO synthase.[2,16] To further explore a molecular basis for the oscillatory release of NO from neurons, we simultaneously visualized the neuronal NO release and the Ca^{2+} dynamics in neurons. We introduced a FRET-based Ca^{2+} indicator, yellow cameleon 3.60,[17] into hippocampal neurons and then cocultured Piccell with the hippocampal neurons. Ca^{2+} spikes were observed spontaneously and periodically in the neurons, as previously reported[18,19] (Figure 3g upper graph). We found that each NO release from neurons synchronized with each repeat of several consecutive Ca^{2+} spikes (Figure 3g). The result indicates that neuronal NO synthase is transiently and repeatedly activated by each repeat of the Ca^{2+} spikes to generate NO periodically. Taken together, we conclude that the oscillatory release of picomolar concentrations of NO is governed by the oscillatory changes of the Ca^{2+} concentrations in hippocampal neurons during spontaneous neurotransmission. There is mounting evidence that NO is a key factor that regulates neuronal functions.[20−22] However, the dynamics of neuronal NO release has remained to be revealed. The oscillatory release of picomolar concentrations of NO from neurons that we found using Piccell may be fundamental to the regulation of neuronal functions such as neurotransmission, learning, and memory. These results thus demonstrate that Piccell provides a powerful tool to analyze the picomolar dynamics of NO release from neurons.

Imaging a Diffusing Process of NO Released from Single Living Cells. Because Piccell is a cell-based indicator, a monolayer culture of Piccell is readily prepared (Figure 4a). This Piccell sheet was incubated with the caged NO. By a local UV irradiation (diameter of 20 μm), we transiently generated NO only in a spatially limited region of a particular Piccell located at the center of the image (Piccell-1, Figure 4a). The local NO generation provoked a transient FRET response of Piccell-1 but not with Piccell-2, which is located away from Piccell-1 in the Piccell sheet (Figure 4a and b). In contrast, when NO was extensively generated by a uniform uncaging of the caged NO, the Piccell sheet exhibited a homogeneous FRET response throughout the image (Figure 4a and b). The local response of the Piccell sheet was reproducible upon repeated local uncaging of the caged NO (Figure 4b; Supporting Information movie). The Piccell sheet thus visualizes the spatiotemporal dynamics of NO on the basis of each independent response of Piccell in the sheet.

Using the Piccell sheet, we visualized a diffusing process of NO from single vascular endothelial cells. The Piccell sheet was prepared on a coverslip. Endothelial cells were separately cultured on a glass-bottomed dish. We gently placed the coverslip on the glass-bottomed dish in the presence of caged ATP, p^3-[1-(2-nitrophenyl)ethyl]adenosine-5′-triphosphate.[23] In this condition, the Piccell sheet faces the endothelial cell on the glass-bottomed dish (Supporting Information Figure 2). We then uncaged the caged ATP with a transient UV irradiation to stimulate the endothelial cell. The transient ATP generation resulted in the transient response of a limited number of Piccells on the endothelial cell (Figure 4c). The peak response of Piccell that directly faces the endothelial cell indicates the release of 100 pM NO from the endothelial cell (region-1 in Figure 4c and d). The response of Piccell in the sheet decreased with distance from the endothelial cell and disappeared in 60 μm. (Figure 4d and e). The result illustrates the diffusion range of NO generated in the endothelial cell upon ATP stimulation. Because NO is a diffusible messenger, it has been a key question how far single NO-donating cells such as vascular endothelial cells and neurons remotely regulate the adjacent cells through the released NO. The Piccell sheet exhibits such information upon visualizing the spatially limited diffusion of released NO from single living cells.

DISCUSSION

In the present study, we have developed a cell-based indicator Piccell. Based on the signal amplification mechanism, Piccell

(14) Wisden, W.; Seeburg, P. H. *Curr. Opin. Neurobiol.* **1993**, *3*, 291−298.
(15) Burnashev, N. *Curr. Opin. Neurobiol.* **1996**, *9*, 311−317.
(16) Sattler, R.; Xiong, Z.; Lu, W.-Y.; Hafner, M.; MacDonald, J. F.; Tymianski, M. *Science* **1999**, *284*, 1845−1848.
(17) Nagai, T.; Yamada, S.; Tominaga, T.; Ichikawa, M.; Miyawaki, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10554−10559.
(18) Abele, A. E.; Scholz, K. P.; Scholz, W. K.; Miller, R. J. *Neuron* **1990**, *2*, 413−419.
(19) Bacci, A.; Verderio, C.; Pravettoni, E.; Matteoli, M. *Eur. J. Neurosci.* **1999**, *11*, 389−397.
(20) Son, H.; Hawkins, R. D.; Martin, K.; Kiebler, M.; Huang, P. L.; Fishman, M. C.; Kandel, E. R. *Cell* **1996**, *87*, 1015−1023.
(21) Micheva, K. D.; Buchanan, J.; Hotz, R. W.; Smith, S. J. *Nat. Neurosci.* **2003**, *6*, 925−932.
(22) Huang, Y.; Man, H.-Y.; Sekine-Aizawa, Y.; Han, Y.; Juluri, K.; Luo, H.; Cheah, J.; Lowenstein, C.; Huganir, R. L.; Snyder, S. H. *Neuron* **2005**, *46*, 533−540.
(23) Walker, J. W.; Reid, G. P.; Trentham, D. R. *Methods Enzymol.* **1989**, *172*, 288−301.

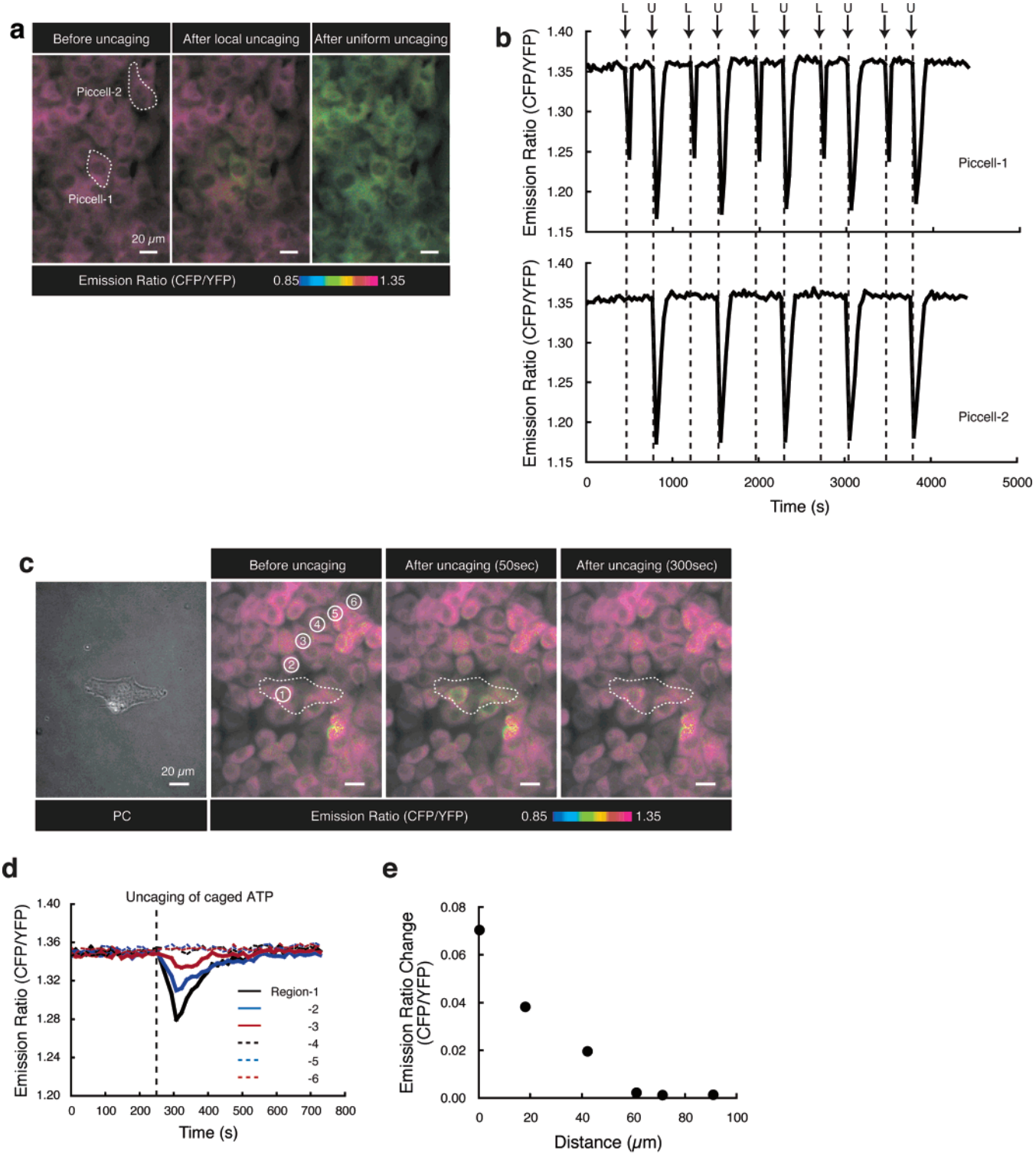

Figure 4. Visualization of a diffusing process of NO from cells with Piccell (a) Pseudocolor images of the CFP/YFP emission ratio of the monolayer culture of Piccell before uncaging (left), after local uncaging (center), and after subsequent uniform uncaging (right) of the caged NO. The local uncaging of the caged NO was performed in the spatially limited region of Piccell-1 by the local UV irradiation in a diameter of 20 μm. (b) Every time NO was generated by local or uniform uncaging of the caged NO, Piccell-1 exhibited a sharp FRET response in the monolayer culture of Piccell (upper panel). Only when NO was generated by the uniform uncaging of the caged NO, Piccell-2 exhibited sharp FRET responses in the monolayer culture of Piccell (lower panel). (c) A phase contrast image of an endothelial cell and pseudocolor images of the CFP/YFP emission ratio of the Piccell sheet before uncaging and 50 and 300 s after uncaging of the caged ATP. Because the Piccell sheet is placed on the endothelial cell on a glass-bottomed dish, the phase contrast image of the endothelial cell and pseudocolor images of the Piccell sheet were separately obtained at each different focus plane. The position of the endothelial cell is shown with broken lines in the pseudocolor images of the Piccell sheet. (d) Time course of the FRET responses of Piccells in region-1, −2, −3, −4, −5, and −6, which are indicated with circles in (c). The caged ATP was uncaged at the time shown with a broken line, and the FRET levels of Piccells in the sheet were monitored. (e) The peak responses of Piccells in region-1, −2, −3, −4, −5, and −6 in (d) were plotted as a function of distance from the endothelial cell to each Piccell.

detected picomolar concentrations of NO release from living cells. It is worth comparing the sensitivity of Piccell with that of the existing organic molecular indicators, such as DAF,[5] DAC,[6] and CuFL.[7] Sasaki et al. have reported that 6 μM NO elicits the approximate half-maximal response of DAC-P, the reaction efficiency of which is at least 53 times higher than that of DAF-2.[6] In contrast, 100 pM NO provokes the half-maximal response of Piccell. On the basis of these results, Piccell has several orders of magnitude superior sensitivity to that of the latest organic fluorescent indicator, DAC-P. Although Lim et al. have reported the copper-based fluorescent indicator CuFL,[7] they have not shown a dose response of the indicator that might have allowed a comparison of Piccell with CuFL as to the sensitivity. In addition to the sensitivity, the organic indicators, such as DAFs, DACs, and CuFL, have no reversibility in their fluorescence responses for NO.[5−7] This is why the organic indicators do not detect the decrease in NO concentrations in the cell. These limitations of the organic indicators as to the sensitivity and reversibility have prevented the organic indicators from uncovering the complicated picomolar dynamics of NO occurring in living cells. The outstanding sensitivity of Piccell and its reversibility have led us to uncover the oscillatory release of picomolar concentrations of NO from hippocampal neurons.

We have recently developed the genetically encoded molecular indicator NOA for the analysis of NO in living cells.[4] NOA was expressed in vascular endothelial cells to analyze the spatiotemporal dynamics of NO in the endothelial cells.[4] Unlike NOA, the present cell-based indicator Piccell was developed for the analysis of NO release from living cells. This is the first major difference between NOA and the present Piccell. Then we made further comparisons between NOA and Piccell in terms of sensitivity, reversibility, experimental feasibility, and so on. Both NOA and Piccell allow high-sensitive detection of NO compared with the organic fluorescent indicators such as DAFs,[5] DACs,[6] and CuFL.[7] This is because both of them are based on signal amplification through the enzymatic generation of cGMP. NOA and Piccell actually exhibited the detection limit of 100 pM NO and that of 20 pM NO, respectively. As to the reversibility of NOA, it is dependent largely on the cell types, in which NOA is introduced for the analysis of NO. Expression of phosphodiesterases that hydrolyze cGMP is a requisite for the reversible response of NOA. NOA is thus reversible in the cell types that express the phosphodiesterases but not in other cell types that do not express them. In contrast, Piccell is superior in reversibility because it is a cloned cell-based indicator that expresses endogenous phosphodiesterases. Also, Piccell is superior in reproducibility and applicable to various cell types under identical conditions in contrast to NOA, response profiles of which are affected by the extent of the phosphodiesterases in the cells to be analyzed. In terms of experimental feasibility, NOA has a limitation that is not true of Piccell. Because NOA is a genetically encoded molecular indicator, efficient introduction and expression in the cell of cDNA encoding NOA is required. However, we often encounter difficulties in gene expression in some cell types including neurons, and NOA is no exception. By contrast, the cell-based indicator Piccell is just cocultured with NO-releasing cells for the analysis of NO from the target cells. Thus, the cell types to be analyzed do not limit the usage of Piccell. In the case of NOA, the NO-induced cGMP generation takes place in the NO-releasing cells that express NOA. In the case of Piccell, the cGMP generation takes place in Piccell but not in the NO-releasing cells. This is also an advantage of Piccell, because the generated cGMP in Piccell never interferes with signaling processes in the NO-releasing cells. In addition, Piccell expands its usage for the simultaneous analysis of NO and other cellular signaling processes associated with the NO dynamics including Ca^{2+}, as shown here. This is because Piccell that is placed outside the NO-releasing cells does not optically interfere with the response of other fluorescent indicators introduced in the cells. Here we actually performed this by using Piccell and the Ca^{2+} indicator and uncovered the missing temporal and quantitative link between NO and Ca^{2+} in hippocampal neurons. By contrast, NOA may suffer from possible spectral overlaps with other fluorescent indicators when introduced in the cell together with the other fluorescent indicators. This prevents us from simultaneously analyzing NO and other cellular signaling processes with NOA and other fluorescent indicators. Piccell has thus overcome the limitations of existing methods including organic and genetically encoded molecular indicators for NO and thereby provides a novel and powerful tool for the analysis of NO release from living cells.

CONCLUSION

We have developed a cell-based indicator to visualize picomolar dynamics of NO released from living cells. The cell-based indicator, named Piccell, exhibited exceptional sensitivity, selectivity, reversibility, and reproducibility. The outstanding sensitivity of Piccell led us to uncover the oscillatory release of picomolar concentrations of NO from hippocampal neurons. Piccell, which is placed outside NO-releasing cells, allows the simultaneous imaging of the released NO and other cellular signaling processes. We revealed the missing temporal and quantitative link between NO and Ca^{2+} in hippocampal neurons. Because Piccell is a living cell-based indicator, a monolayer culture of Piccell is readily prepared. Using this Piccell sheet, we visualized the extent of NO diffusion from single vascular endothelial cells. Piccell thus provides a powerful tool to uncover picomolar dynamics of NO that regulates a wide range of cell functions in biological systems.

ACKNOWLEDGMENT

This work has been supported by grants from Japan Science and Technology Agency, the Ministry of Education, Science and Culture, Takeda Science Foundation (to M.S.), and Sumitomo Foundation (to M.S.). We thank Dr. N. Hida for helpful discussions.

Received for review September 22, 2006. Accepted October 15, 2006.

AC061791B