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Published on Web 05/17/2002

Improvement and Biological Applications of Fluorescent Probes for Zinc, ZnAFs

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Received January 11, 2002

Abstract: The development and cellular applications of novel fluorescent probes for Zn^{2+}, ZnAF-1F, and ZnAF-2F are described. Fluorescein is used as a fluorophore of ZnAFs, because its excitation and emission wavelengths are in the visible range, which minimizes cell damage and autofluorescence by excitation light. *N,N*-Bis(2-pyridylmethyl)ethylenediamine, used as an acceptor for Zn^{2+}, is attached directly to the benzoic acid moiety of fluorescein, resulting in very low quantum yields of 0.004 for ZnAF-1F and 0.006 for ZnAF-2F under physiological conditions (pH 7.4) due to the photoinduced electron-transfer mechanism. Upon the addition of Zn^{2+}, the fluorescence intensity is quickly increased up to 69-fold for ZnAF-1F and 60-fold for ZnAF-2F. Apparent dissociation constants (K_d) are in the nanomolar range, which affords sufficient sensitivity for biological applications. ZnAFs do not fluoresce in the presence of other biologically important cations such as Ca^{2+} and Mg^{2+}, and are insensitive to change of pH. The complexes with Zn^{2+} of previously developed ZnAFs, ZnAF-1, and ZnAF-2 decrease in fluorescence intensity below pH 7.0 owing to protonation of the phenolic hydroxyl group of fluorescein, whose pK_a value is 6.2. On the other hand, the Zn^{2+} complexes of ZnAF-1F and ZnAF-2F emit stable fluorescence around neutral and slightly acidic conditions because the pK_a values are shifted to 4.9 by substitution of electron-withdrawing fluorine at the ortho position of the phenolic hydroxyl group. For application to living cells, the diacetyl derivative of ZnAF-2F, ZnAF-2F DA, was synthesized. ZnAF-2F DA can permeate through the cell membrane, and is hydrolyzed by esterase in the cytosol to yield ZnAF-2F, which is retained in the cells. Using ZnAF-2F DA, we could measure the changes of intracellular Zn^{2+} in cultured cells and hippocampal slices.

Introduction

Zinc ion (Zn^{2+}) is an essential component of many enzymes and transcription factors (e.g., carbonic anhydrase, zinc finger proteins, etc.), in which it plays structural or catalytic roles.[1] In addition to such protein-bound Zn^{2+}, free or loosely bound (labile, chelatable) Zn^{2+} exists at high concentration especially in brain,[2] pancreas,[3] and spermatozoa.[4] In brain, a few millimolar of free Zn^{2+} exists in the vesicles of presynaptic neurons, and is released by synaptic activity or depolarization, modulating the function of certain ion channels and receptors. Zn^{2+} has been reported to induce selective neuronal cell death that is associated with certain acute conditions, including epilepsy,[5] transient global ischemia,[6] and brain injury.[7] Although many reports describe the significance of Zn^{2+} in biological systems, its mechanisms of action are poorly understood.

Fluorescent probes, which allow visualization of cations, small molecules, or enzyme activity in living cells by fluorescence microscopy, are useful tools for clarifying the function in biological systems.[8] Therefore, various fluorescent probes for Zn^{2+} have been developed.[9−11,13−16] The most widely used fluorescent probes for Zn^{2+} are TSQ[9] (6-methoxy-8-(*p*-tolu-

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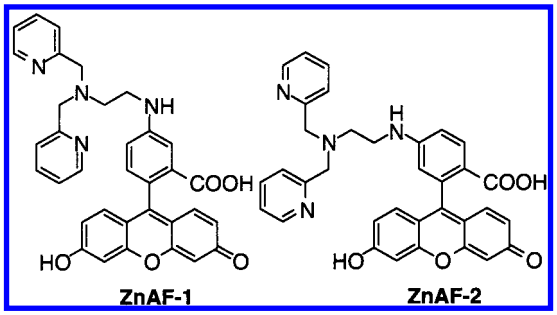

Figure 1. Structures of the fluorescent probes for Zn^{2+}: ZnAF-1 and ZnAF-2.

enesulfonamide)quinoline) and its derivatives.[3,10,11] One of these derivatives, Zinquin, can detect intracellular Zn^{2+} in living cells.[3,10] Although quinoline-based probes are useful, they are not ideal because the excitation wavelength is in the ultraviolet range, which may cause cell damage and is subject to interference by autofluorescence from biological molecules such as pyridine nucleotides. Fluorescein is the most widely used fluorophore for labeling and sensing biomolecules,[12] because it has a high extinction coefficient and a high fluorescence quantum yield in aqueous solution, and its excitation wavelength is in the visible range, which is preferable to UV excitation. Recently, fluorescein-based probes, Zinpyr-1 and Zinpyr-2,[13] which fluoresce in the presence of Zn^{2+}, have been reported. However, the basal fluorescence of Zinpyrs is pH-sensitive around neutral conditions with pK_a values of 8.4 for Zinpyr-1 and 9.4 for Zinpyr-2, and its intensity is strong (quantum yield is 0.39 for Zinpyr-1 and 0.25 for Zinpyr-2) at pH 7.0, so cellular pH change induced by biological stimuli[17] can cause difficulty in interpreting fluorescence intensity change. Therefore, Zinpyr-1 and Zinpyr-2 cannot reliably monitor Zn^{2+} in living cells. We previously reported fluorescein-based probes, ZnAF-1 and ZnAF-2[14] (Figure 1). Upon addition of Zn^{2+}, the fluorescence intensity was increased by 17-fold for ZnAF-1 and 51-fold for ZnAF-2 at pH 7.5. At this pH, the fluorescence intensity of ZnAF-1 or ZnAF-2 itself is very small; the quantum yield is only 0.02 for both ZnAFs, and is not increased by pH change. However, the fluorescence intensity of the Zn^{2+} complex with ZnAF-1 or ZnAF-2 is decreased below pH 7.0. So, although ZnAF-1 or ZnAF-2 is useful above pH 7.0, the signal is affected, for example, under conditions of acidosis.

Here we report the design and synthesis of fluorine-substituted derivatives of ZnAFs, ZnAF-1F, and ZnAF-2F, whose complexes with Zn^{2+} exhibit stable fluorescence above pH 6.0, allowing detection of Zn^{2+} regardless of physiological pH changes in living cells. We also report the fluorescence and kinetic properties of these ZnAFs and describe biological applications in cultured cells and hippocampal slices.

Results and Discussion

Design and Synthesis of ZnAF-1F and -2F. The fluorescence intensity of fluorescein, used as a fluorophore of ZnAF-1 and ZnAF-2, decreases under acidic conditions. This property arises from protonation of the phenolic hydroxyl group of fluorescein, whose pK_a value is 6.43.[18] The Zn^{2+} complex of ZnAF-1 or ZnAF-2 exhibits similar properties, and the pK_a values of these ZnAFs are almost the same, 6.2. So, we designed ZnAF-1F and ZnAF-2F with electron-withdrawing fluorine at the ortho position of the phenolic hydroxyl group to lower the pK_a value, and thereby obtain stable fluorescence under near-neutral conditions.

The synthetic scheme for ZnAF-1F and ZnAF-2F is shown in Scheme 1. 2′,7′-Difluoro-5- and 6-nitrofluorescein were formed from 4-nitrophthalic acid anhydride and 4-fluororesorcinol by heating in methanesulfonic acid. Then the nitro group was reduced to an amino group with Na_2S and NaSH in water, as in the synthesis of aminofluorescein.[19] The phenolic hydroxyl group of fluorescein was protected as the pivaloate ester. Direct alkylation of the nitrogen atom of aminofluorescein dipivaloate ester (**1**) was difficult because of its low electron density, so the amino group was converted to 4-nitrobenzenesulfonamide. At this stage, the 5-isomer (**2**) and 6-isomer (**2′**) could be easily separated by silica gel chromatography. Then, reaction with 1,2-dibromoethane and 2,2′-dipicolylamine afforded **4**. 4-Nitrobenzenesulfonamide was converted to the secondary amine and the pivaloate ester was cleaved to yield ZnAF-1F. The 6-substituted isomer ZnAF-2F was also similarly obtained from **2′**.

Derivatives of fluorescein that are amino-substituted at the benzoic acid moiety exhibit almost no fluorescence, but when the amino group is converted to a less electron-donating group, such as an amide group, or complexed with a cation via the lone pair of nitrogen, fluorescence with a high quantum yield is obtained. If we can design and synthesize a suitably specific reactive moiety, which is linked to aminofluorescein, we should be able to obtain sensor molecules suitable for different types of analytes. It is also advantageous that the fluorescence quantum yield of aminofluorescein is very low, so if the sensor molecule is converted to fluorescent form, the measured fluorescence intensity will be mainly due to the analyte. For such a strategy, **2** and **2′** could be good intermediates, because 4-nitrobenzenesulfonamide can be alkylated efficiently with alcohol or alkyl halide, and deprotected under mild conditions.[20]

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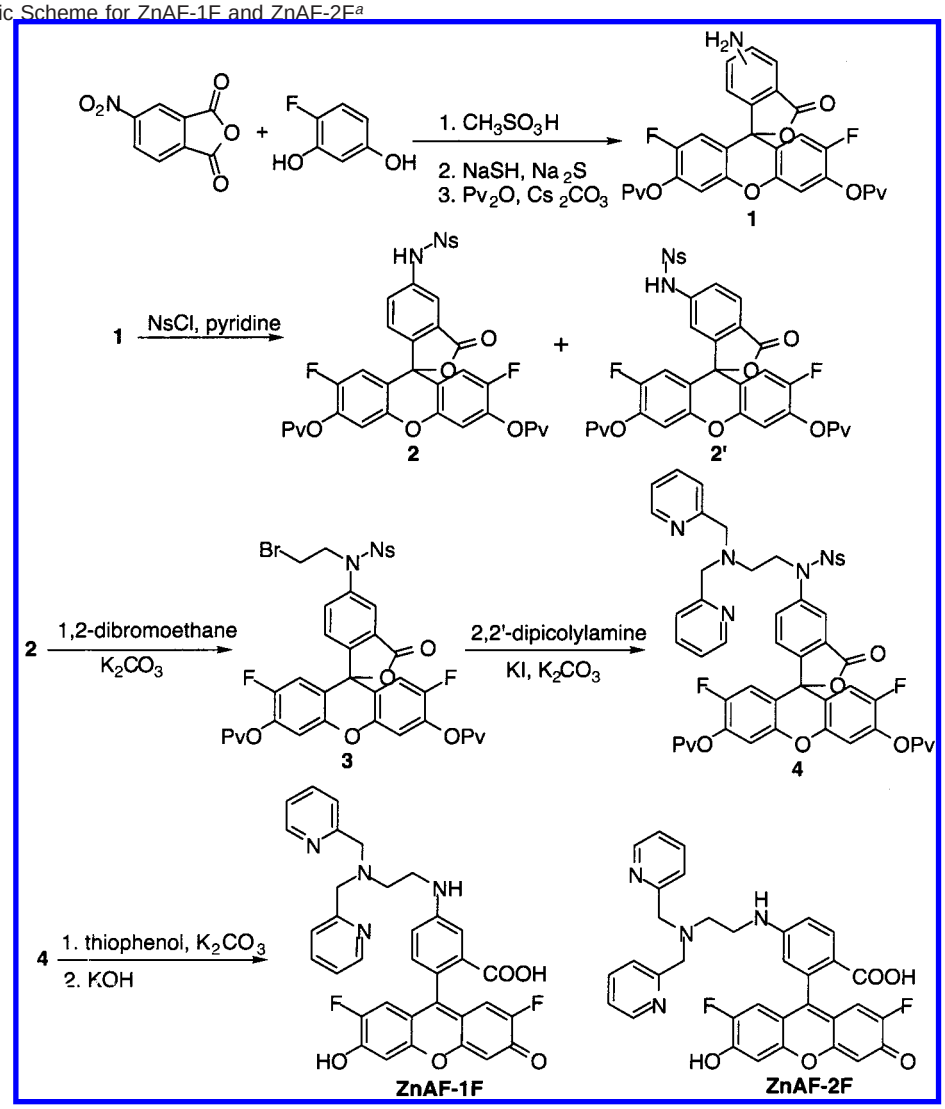
Scheme 1. Synthetic Scheme for ZnAF-1F and ZnAF-2F[a]



[a] Ns = 4-nitrobenzenesulfonyl, Pv = Pivaloyl.

Table 1. Spectroscopic Properties of ZnAFs with and without Zn^{2+} [a]

	free		+Zn^{2+}	
compd	ϵ^b	Φ^c	ϵ^b	Φ^c
ZnAF-1	7.4×10^4 (489)	0.022	6.3×10^4 (492)	0.21
ZnAF-2	7.8×10^4 (490)	0.023	7.6×10^4 (492)	0.32
ZnAF-1F	7.7×10^4 (489)	0.004	7.0×10^4 (492)	0.17
ZnAF-2F	7.4×10^4 (490)	0.006	7.3×10^4 (492)	0.24

[a] All data were obtained at pH 7.4 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)). [b] ϵ stands for extinction coefficient (M^{-1} cm^{-1}). Measured at each λ_{max}, which is shown in parentheses (nm). [c] Φ stands for quantum yield, determined using Φ of fluorescein (0.85) in 0.1 N NaOH as a standard.[21]

Fluorescence Properties of ZnAF-1F and -2F. ZnAF-1F and ZnAF-2F themselves showed almost no fluorescence under physiological conditions (pH 7.4, $I = 0.1$ ($NaNO_3$)), exhibiting fluorescence quantum yields of only 0.004 for ZnAF-1F and 0.006 for ZnAF-2F (Table 1). These values are much lower than those of existing Zn^{2+} fluorescent probes, Zinpyr-1 (0.38), Zinpyr-2 (0.25), ZnAF-1 (0.02), and ZnAF-2 (0.02), suggesting that ZnAF-1F and ZnAF-2F will exhibit very low background fluorescence. Upon addition of Zn^{2+}, the fluorescence intensity was increased by 69-fold for ZnAF-1F and 60-fold for ZnAF-2F, and the quantum yield of the Zn^{2+} complex was 0.17 for ZnAF-1F and 0.24 for ZnAF-2F. The absorption maximum wavelength was very slightly bathochromically shifted (2−3 nm). The emission maximum did not change upon the addition of Zn^{2+} for either ZnAF-1F or ZnAF-2F (Figure 2 and Table 1).

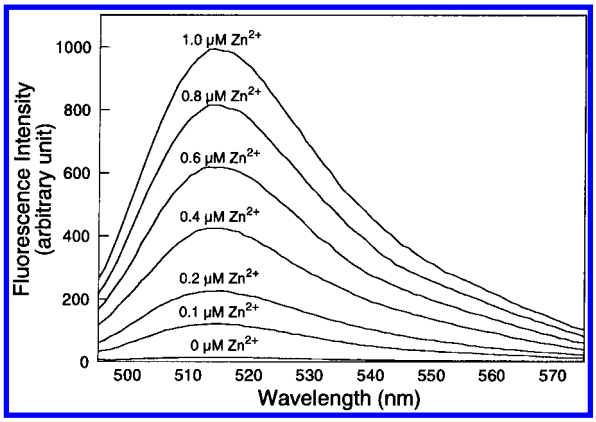


Figure 2. Emission spectra (excitation at 492 nm) of 1 μM ZnAF-2F in the presence of various concentrations of Zn^{2+} ranging from 0 to 1 μM. These spectra were measured at pH 7.4 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)).

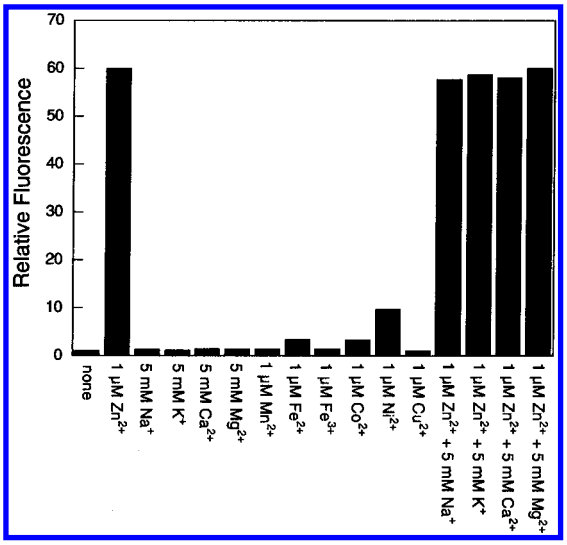


Figure 3. The relative fluorescence intensity of 1 μM ZnAF-2F in the presence of various cations. These data were measured at pH 7.4 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)).

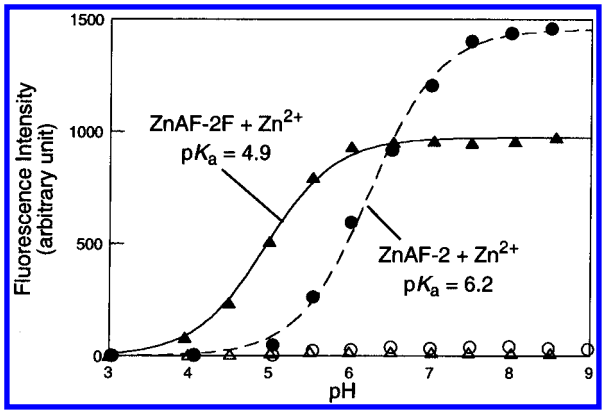


Figure 4. Effect of pH on the fluorescence intensity of ZnAF-2 and ZnAF-2F: open circle, 1 μM ZnAF-2; closed circle, 1 μM ZnAF-2 + 1 μM Zn^{2+}; open triangle, 1 μM ZnAF-2F; closed triangle, 1 μM ZnAF-2F + 1 μM Zn^{2+}. These data were fitted to the following equations: ZnAF-2F + Zn^{2+} (shown as a solid line), $F = 973/(1 + 10^{4.9-pH})$, $R = 1.00$; ZnAF-2 + Zn^{2+} (shown as a broken line), $F = 1456/(1 + 10^{6.2-pH})$, $R = 1.00$. F = fluorescence intensity.

Other cations which exist at high concentration in living cells, Ca^{2+}, Mg^{2+}, Na^+, and K^+, did not enhance the fluorescence intensity even at high concentration (5 mM), as shown in Figure 3. These results are presumably due to the poor complexation of alkaline metals or alkaline earth metals with the chelator of ZnAFs, based on the absence of any obvious change of UV−visible spectrum upon addition of these cations, which also did not interfere with the Zn^{2+}-induced fluorescence enhancement. Among first-row transition metal cations, Fe^{2+}, Co^{2+}, and Ni^{2+} induced a slight enhancement of the fluorescence intensity, and Cu^{2+} quenched the fluorescence. Like TPEN (*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine), ZnAFs probably form complexes with these transition metal cations, but the fluorescence is weakened because of an electron or energy transfer between metal cation and fluorophore, which is known as the fluorescence quenching mechanism.[22]

The Effect of pH on the Fluorescence Intensity. The fluorescence intensity of the Zn^{2+} complex with ZnAF-2 decreased below pH 7.0, whereas that of ZnAF-2F hardly changed above pH 6.0 (Figure 4). The pK_a values of the phenolic hydroxyl group of fluorescein were calculated to be 6.2 for both ZnAF-1 and ZnAF-2, and 4.9 for both ZnAF-1F and ZnAF-2F. These shifts of pK_a presumably arise from the fluorine substitution. So, the fluorescence intensity of the Zn^{2+} complex with ZnAF-1F or ZnAF-2F is not subject to interference by pH changes under near-neutral or slightly acidic conditions.

The fluorescence intensity of ZnAF-1F or ZnAF-2F in the absence of Zn^{2+} did not change above pH 5.0. Fluorescence properties of fluorescein derivatives are believed to be controlled by a photoinduced electron transfer (PET) process from the benzoic acid moiety to the xanthene ring, and fluorescence on/off switching should depend on the highest occupied molecular orbital (HOMO) levels of the benzoic acid moiety.[12d] The enhancement of fluorescence intensity of ZnAFs might be controlled by the coordination of nitrogen directly attached to the benzoic acid moiety by proton or metal cations, resulting in a change of HOMO levels. The pK_a value of this pivotal nitrogen for fluorescence seems to be as low as 5.0, since ZnAFs do not fluoresce upon protonation above this pH. Under strongly acidic conditions this nitrogen would be protonated, but the fluorescence would be quenched because the phenolic hydroxyl group of fluorescein would also be protonated. This insensitivity to pH of the fluorescence is extremely useful for applications to living cells, where pH changes are caused by certain biological stimuli.[17]

Kinetic Analysis of the Complex Formation with Zn^{2+}. Upon addition of various concentrations of Zn^{2+}, the fluorescence intensity of ZnAF (1 μM) linearly increased up to a 1:1 [ZnAF]/[Zn^{2+}] ratio, and the fluorescence and absorption spectra did not change between 1 and 100 μM Zn^{2+} addition. Furthermore, a Job's plot analysis revealed maximum fluorescence obtained at a 1:1 ratio. These data suggested that ZnAF should form a 1:1 complex with Zn^{2+}. So, the apparent dissociation constants, K_d, were determined from the fluorescence intensity in 100 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) buffer (pH 7.4, $I = 0.1$ ($NaNO_3$)) with 10 mM NTA (nitrilotriacetic acid) and 0−9 mM $ZnSO_4$ at 25 °C. The fluorescence intensity data (Figure 5) were fitted to eq 1, and K_d was calculated,

$$F = F_0 + (F_{max} - F_0)\frac{[Zn^{2+}]_f}{K_d + [Zn^{2+}]_f} \quad (1)$$

where F is the fluorescence intensity, F_{max} is the maximum fluorescence intensity, F_0 is the fluorescence intensity in the absence of Zn^{2+}, and $[Zn^{2+}]_f$ is the free Zn^{2+} concentration calculated according to the reported method.[14,22] The calculated constants shown in Table 2 suggested that ZnAFs can quantitatively measure the concentration of Zn^{2+} around the nanomolar range (0.1−10 nM order) as shown in Figure 5, which is sufficient sensitivity for application in mammalian cells. Above this range, they maximally fluoresced, and can detect the change of Zn^{2+} qualitatively, although the precise concentration cannot be determined. And the values of K_d almost did not change with the addition of Ca^{2+} or Mg^{2+} (total concentration: 1 mM). The

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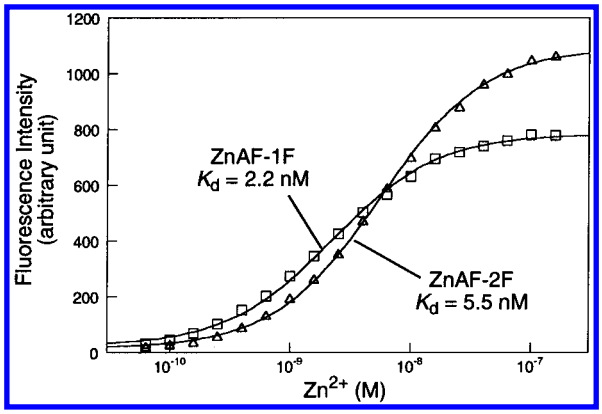

Figure 5. Fluorescence intensity of 1 μM ZnAF-1F (open square) or ZnAF-2F (open triangle) as a function of the concentration of free Zn^{2+} in 100 mM HEPES buffer (pH 7.4, I = 0.1 ($NaNO_3$)) with 10 mM NTA (Nitrilotriacetic acid) and 0−9 mM Zn^{2+} at 25 °C. These data were fitted to eq 1 (shown as a solid line) and K_d was calculated.

Table 2. Apparent Dissociation Constants (K_d) and Association and Dissociation Rate Constants (k_{on} and k_{off}) of ZnAFs in 100 mM HEPES Buffer (pH 7.4, I = 0.1 ($NaNO_3$)) at 25 °C

compd	K_d (nM)	k_{on}[a] ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
ZnAF-1	0.78	4.3×10^6	3.4×10^{-3}
ZnAF-2	2.7	3.1×10^6	8.4×10^{-3}
ZnAF-1F	2.2	3.5×10^6	7.7×10^{-3}
ZnAF-2F	5.5	3.2×10^6	1.8×10^{-2}

[a] These data were measured under pseudo-first-order conditions, final concentrations: 1 μM ZnAFs; 50 μM $ZnSO_4$.

K_d of ZnAF-1F and ZnAF-2F were slightly lower than that of ZnAF-1 or ZnAF-2, probably because of the electron-withdrawing property of fluorine.

Next, we measured the association (k_{on}) and dissociation (k_{off}) rate constants of ZnAF-1, ZnAF-2, ZnAF-1F, and ZnAF-2F at pH 7.4 (100 mM HEPES buffer, I = 0.1 ($NaNO_3$)), 25 °C using a stopped-flow spectrofluorimeter. The rate constants of the ZnAF−Zn^{2+} complex formation are given by:

$$\frac{d[ZnAF - Zn^{2+}]}{dt} = k_{on}[ZnAF][Zn^{2+}] - k_{off}[ZnAF - Zn^{2+}] \quad (2)$$

In this experiment, the final concentration of Zn^{2+} (50 μM) is much higher than that of ZnAF (1 μM), so eq 2 can be approximated to

$$\frac{d[ZnAF - Zn^{2+}]}{dt} = k_{on}'[ZnAF] - k_{off}[ZnAF - Zn^{2+}] \quad (3)$$

where $k_{on}' = k_{on}[Zn^{2+}]$. Thus,

$$\ln\left(\frac{F_{max} - F}{F_{max} - F_0}\right) = -k_{obs}t \quad (4)$$

$$F = F_{max} - (F_{max} - F_0)\exp(-k_{obs}t) \quad (5)$$

where k_{obs} is $k_{on}' + k_{off}$, F is the fluorescence intensity, F_{max} is the maximum fluorescence intensity, and F_0 is the fluorescence intensity in the absence of Zn^{2+}.

The time-dependent fluorescence intensity data (Figure 6) were fitted to eq 5, and k_{obs} was obtained. Then, k_{on} was calculated by using eq 6, and k_{off} was calculated by using eq 7.

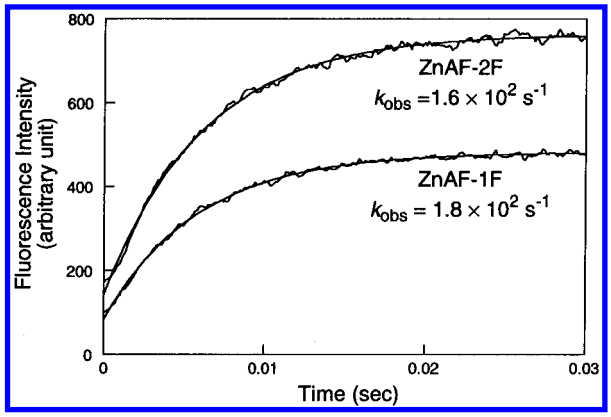

Figure 6. Time course measurement of the fluorescence intensity. ZnAF mixed with Zn^{2+} (final concentration: 1 μM ZnAF; 50 μM Zn^{2+}) at pH 7.4 (100 mM HEPES buffer, I = 0.1 ($NaNO_3$)) and 25 °C. These data were fitted to eq 5 (shown as a solid line) and k_{obs} was calculated.

$$k_{on} = \frac{k_{obs}}{[Zn^{2+}] + K_d} \quad (6)$$

$$k_{off} = K_d k_{on} \quad (7)$$

The calculated values of these constants are shown in Table 2. The k_{on} values of ZnAFs were almost the same, (3∼4) × 10^6 M^{-1} s^{-1}, implying that the complexation is sufficiently fast to detect an increase of Zn^{2+} concentration within a few hundred milliseconds. These values are 2 orders of magnitude smaller than those of complexation of Ca^{2+} with fluorescent probes for Ca^{2+} such as fura-2, indo-2 and quin-2,[24] possibly because of the nature of the metal cations, for example, the water exchange rate constant, which correlates with association rates, of Zn^{2+} (3×10^7 s^{-1}) is smaller than that of Ca^{2+} (10^8 s^{-1}). Although the values of k_{off} are relatively small, this is inevitable for high-affinity probes such as ZnAFs, whose K_d values are in the nanomolar range, and the dissociation rates are considered to be not too slow for the reversible assay of Zn^{2+} concentration. If other probes, with different properties, were needed, they could be easily synthesized from compound **2** or **2′** and a suitable chelating group. For example, the probe, whose acceptor for Zn^{2+} is *N*-(2-pyridylmethyl)ethylenediamine instead of *N*,*N*-bis(2-pyridylmethyl)ethylenediamine, was synthesized via a similar synthetic route. It had almost the same k_{on} value as ZnAFs and a 5 orders of magnitude larger K_d, so that its dissociation rate was sufficiently fast (data not shown).

Biological Applications of ZnAFs. We examined the application of ZnAF-2F to cultured cells or hippocampal slices. Cultured macrophages (RAW 264.7) were incubated with ZnAF-2F for dye loading, but the cells did not stain, indicating that ZnAF-2F could not permeate through the cell membrane (data not shown). So, we newly synthesized a diacetyl derivative, ZnAF-2F DA. ZnAF-2F DA is more lipophilic, and so was expected to permeate well into cells and then be transformed to ZnAF-2F by esterase in the cytosol, where the dye would be

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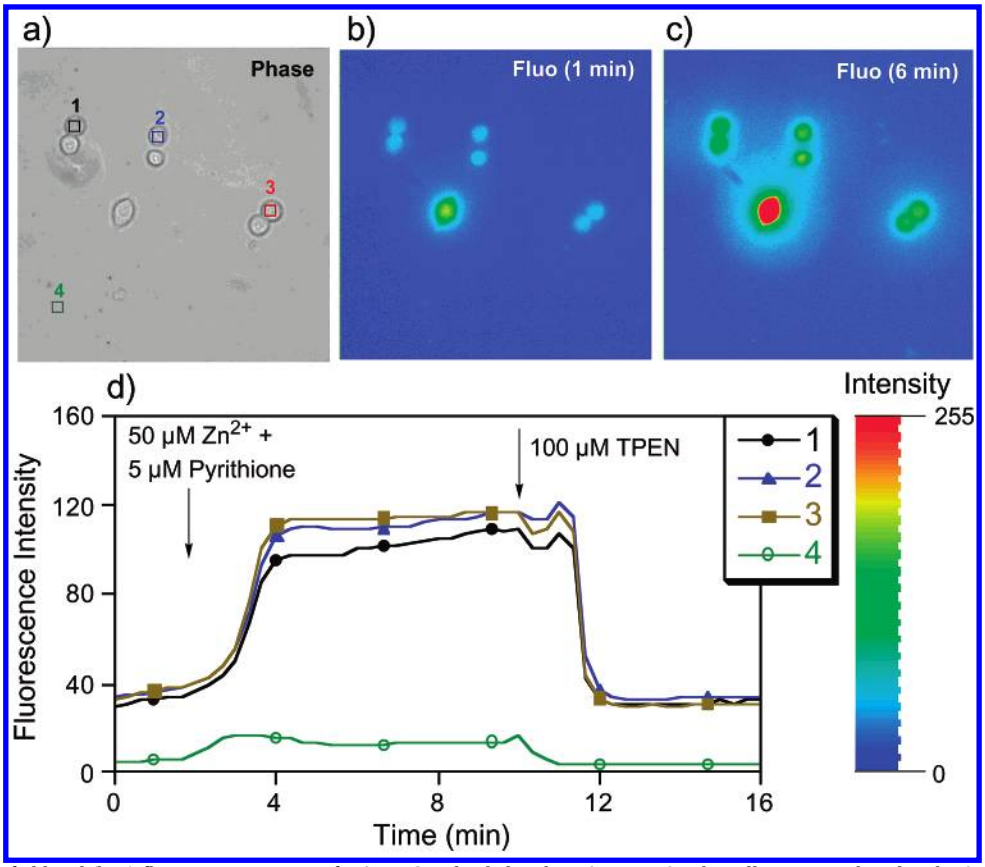

Figure 7. (a) Bright-field and (b, c) fluorescence images of RAW 264.7 loaded with ZnAF-2F DA. The cells were incubated with 10 μM ZnAF-2F DA for 0.5 h at 37 °C. Then the cells were washed with PBS and fluorescence excited at 470−490 nm was measured at 20 s intervals. At 2 min, 5 μM pyrithione and 50 μM $ZnSO_4$ were added to the medium, and 100 μM TPEN was added at 10 min. Fluorescence images are shown in pseudo-color and correspond to the fluorescence intensity data in (d), which showed the average intensities of the corresponding areas (1−3, intracellular region; 4, extracellular region).

retained for a long time. The cells were incubated with 10 μM ZnAF-2F DA for 30 min, which was a sufficient time for intracellular accumulation of ZnAF-2F judging from the weak fluorescence seen in the intracellular regions (Figure 7b). After washing the stained cells, we added Zn^{2+} (50 μM) and the Zn^{2+} ionophore, pyrithione (2-mercaptopyridine *N*-oxide, 5 μM), to the medium at 2 min, which induces an increase of intracellular Zn^{2+}. As a result, the fluorescence in intracellular regions (Figure 7d, 1−3) was increased, although that in extracellular regions (Figure 7d, 4) almost did not change. This fluorescence was decreased by extracellular addition of the cell-permeable transition metal chelator TPEN (100 μM) at 10 min. These results suggested that ZnAF-2F DA can be used to monitor changes of intracellular Zn^{2+}, and should therefore be useful for clarifying the role of Zn^{2+} in biological processes in which the intracellular concentration of Zn^{2+} is important, for example, cell death induced by ischemia.[6]

In addition to the cultured cell systems, we applied ZnAF-2F DA to hippocampal slices. Acute rat hippocampal slices were incubated with 10 μM ZnAF-2F DA for 1.5 h at room temperature for dye loading. The fluorescent image of a dye-loaded slice is shown in Figure 8a. The fluorescence was dense in the hilus and the stratum lucidum of CA3. This distribution is also detected by other staining methods, such as TSQ,[9b] where Zn^{2+} is concentrated in the vesicles, which is numerous in mossy fiber synapses at the CA3 region. The fluorescence was quenched by incubation of the slices with 150 μM TPEN for 30 min, as shown in Figure 8b. Thus, ZnAF-2F DA can be used to detect intracellular free Zn^{2+} in hippocampal slices.

Conclusion

We have developed fluorescent probes for Zn^{2+}, ZnAF-1, ZnAF-2, ZnAF-1F, and ZnAF-2F by utilizing *N*,*N*-bis(2-pyridylmethyl)ethylenediamine as an acceptor for Zn^{2+} and fluorescein as a fluorophore. The selectivity for Zn^{2+} is very high, and fluorescence is not induced by other biologically important cations such as Ca^{2+} or Mg^{2+}. The fluorescence intensity of ZnAFs in the absence of Zn^{2+} was small and was affected by pH change. The complex with Zn^{2+} of ZnAF-1F or ZnAF-2F emitted stable fluorescence under near-neutral and slightly acidic conditions, which is favorable for detecting Zn^{2+} quantitatively in living cells. By the incubation of cultured cells or hippocampal slices with a cell-permeable derivative, ZnAF-2F DA, which is hydrolyzed to ZnAF-2F in the cytosol, intracellular Zn^{2+} concentration, could be monitored. In addition to the intracellular studies, extracellular Zn^{2+} concentration could be detected by the addition of ZnAF-2F to the medium, because ZnAF-2F does not permeate through the cell membrane. This property should be useful especially for neuroscience experiments, because extracellular Zn^{2+} modulates the function of ion channels or receptors, and its effect is dependent on the concentration.[2] ZnAFs have appropriate sensitivity and association rate constants for studies on the biological functions of Zn^{2+}.

Experimental Section

General Information. All reagents and solvents were of the highest commercial quality and were used without purification. ZnAF-1 and

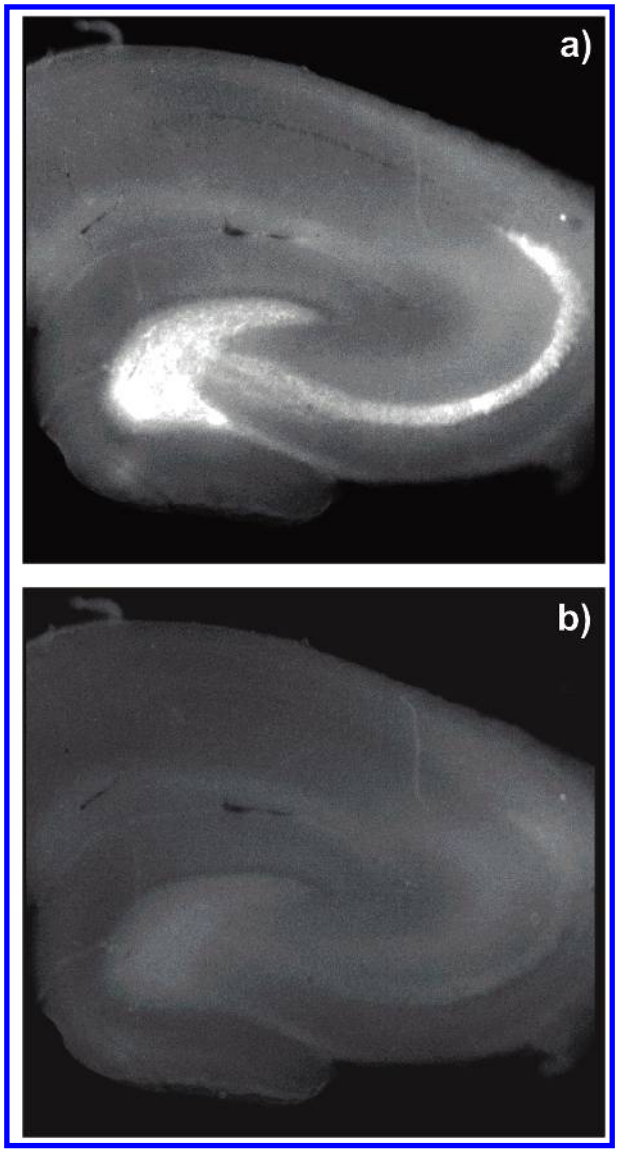

Figure 8. Fluorescence images of ZnAF-2F DA loaded rat hippocampus slices. The slices were incubated with 10 μM ZnAF-2F DA for 1.5 h at room temperature. Then the slices were washed with ACSF for 0.5 h and fluorescence images excited at 470−490 nm were measured (a) before and (b) after incubation with 150 μM TPEN for 0.5 h at room temperature.

ZnAF-2 were prepared as described previously.[14] ZnAF-1F, ZnAF-2F, and ZnAF-2F DA were prepared as described in the Supporting Information.

Fluorometric Analysis. Fluorescence spectroscopic studies were performed with an Hitachi F4500 (Tokyo, Japan). The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. ZnAFs were dissolved in DMSO (for fluorometric analysis; Dojindo, Kumamoto, Japan) to obtain 10 mM stock solutions. Relative quantum yields of fluorescence were obtained by comparing the area under the corrected emission spectrum of the test sample at 492 nm excitation with that of a solution of fluorescein in 0.1 M NaOH (quantum yield: 0.85).[24] UV−visible spectra were measured with a Shimadzu UV-1600 (Tokyo, Japan).

Effect of pH on the Fluorescence Intensity. The following buffers were prepared: 100 mM $Cl_2CHCOOH$−$Cl_2CHCOONa$ buffer (pH 2.0), 100 mM $ClCH_2COOH$−$ClCH_2COONa$ buffer (pH 3.0), 100 mM AcOH−AcONa buffer (pH 4.0−5.0), 100 mM MES (2-morpholinoethanesulfonic acid) buffer (pH 5.5−6.5), 100 mM HEPES buffer (pH 7.0−8.0), and 100 mM CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) buffer (pH 8.5).[25] The fluorescence intensity (excitation 492 nm; emission 514 nm) of samples diluted with these buffers was measured.

Stopped-Flow Measurements. Stopped-flow experiments were performed with a SF-61 DX2 double-mixing stopped-flow spectrofluorimeter (Hi-Tech, Salisbury, UK) equipped with a monochromator between the 75 W xenon light source and the reaction cuvette excitation window. The quartz sample cuvette and syringes containing the reactants were maintained at 25 ± 0.1 °C by a circulating water bath. The pneumatic cylinder was driven by a nitrogen pressure of 6 bar, which resulted in an instrument dead-time of 1 ms. A solution of 2 μM ZnAF in 100 mM HEPES buffer (pH 7.4, $I = 0.1$ ($NaNO_3$)) was combined with an equal volume of 100 μM $ZnSO_4$. Fluorescence was measured with excitation at 492 nm and with emission light (>530 nm), using a 530 cutoff filter.

Imaging System. The imaging system was comprised of an inverted fluorescence microscope (IX-70, Olympus, Tokyo, Japan), cooled charge-coupled device (cooled CCD), camera (Hamamatsu Photonics, Hamamatsu, Japan), and an image processor (Argus 50, Hamamatsu Photonics). The microscope was equipped with a xenon lamp, an objective lens (×40 (RAW 264.7) or ×4 (hippocampus slice)), an excitation filter (470−490 nm), a dichroic mirror (505 nm), and an emission filter (515−550 nm).

Preparation of Cells. RAW 264.7 mouse macrophage cells were kindly provided by Prof. M. Iino (Graduate School of Medicine, The University of Tokyo). Cells were passed in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% fetal bovine serum (JRH Bioscience), 1% penicillin (Gibco BRL), and 1% streptomycin (Gibco BRL) at 37 °C in a 5% CO_2/95% air incubator. Cells were passed 12 h before dye loading onto a glass-bottomed dish at the density of 3×10^5 cells/mL. Then the cells were rinsed with PBS (phosphate-buffered saline, Sigma Diagnostics) and incubated with PBS containing 10 μM ZnAF-2F DA for 30 min at 37 °C. The cells were then washed with PBS twice, and mounted on a microscope stage.

Preparation of Rat Hippocampal Slices. The whole brains of adult Wistar rats (male, 200−250 g) were removed quickly under ether anesthesia and placed in ice-cold ACSF (artificial cerebrospinal fluid), which was aerated with 95% O_2/5% CO_2. The composition of ACSF was 124 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM glucose. The hippocampus was isolated, placed on an agar plate, and sliced into 300 μm thick slices with a rotary slicer (Model DTY 7700, Dosaka Co., Ltd., Osaka, Japan). The fresh hippocampal slices were incubated in ACSF equilibrated with 95% O_2/5% CO_2 for more than 30 min at room temperature. Then they were loaded with 10 μM ZnAF-2F DA for 1.5 h at room temperature. Postincubation with ACSF for 30 min was carried out to wash out the extracellular dye. A small flow-through chamber, whose base consisted of a thin glass coverslip, was placed on a microscope stage. Then, a dye-loaded slice was placed in the chamber and continuously perfused (2.5 mL/min) with equilibrated ACSF at 33−34 °C. The slice was held in place by a metal wire ring with a stretched nylon net.

Acknowledgment. We thank Prof. K. Yamaguchi and Mr. S. Sakamoto of Chiba University for HRMS measurement. We are also grateful to Prof. Y. Kudo, Mr. Y. Kawamura, and Ms. N. Matsumoto of the Tokyo University of Pharmacy and Life Science for advice on the preparation of rat hippocampus slices, and to Prof. M. Iino of The University of Tokyo for donating RAW 264.7. This work was supported in part by the Ministry of Education, Science, Sports and Culture of Japan (Grant Nos. 11794026, 12470475, 12557217, 11771467, and 12045218), by

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the Mitsubishi Foundation, and by the Research Foundation for Opt-Science and Technology. T.H. thanks the Japan Society for the Promotion of Science for a JSPS Research Fellowship for Young Scientists. K.K. is grateful for financial support from Nissan Science Foundation, Shorai Foundation of Science and Technology, and Uehara Memorial Foundation.

Supporting Information Available: Synthetic details and characterization of ZnAF-1F, ZnAF-2F, and ZnAF-2F DA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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