# Communications

#### Fluorescent Probes

## Sodium-Ion-Selective Two-Photon Fluorescent Probe for In Vivo Imaging\*\*

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#### In memory of Chi Sun Hahn

The sodium ion plays critical roles in many physiological and pathological processes such as nerve conduction and muscle and heart contraction; it modulates the electrolyte level, cation transport, and cell volume.<sup>[1,2]</sup> In the mammalian body, most Na<sup>+</sup> ions reside in the extracellular compartment, with much less Na<sup>+</sup> inside cells. The concentration of intracellular free Na<sup>+</sup> ([Na<sup>+</sup>]<sub>i</sub>) is in the range of 5–30 mM, whereas that of extracellular Na<sup>+</sup> is several times higher (greater than 100 mM).<sup>[1]</sup> The influx of Na<sup>+</sup> into a resting cell is regulated by Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX), Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE), and Na<sup>+</sup> channels, whereas the efflux is mediated by the activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase, which transports three Na<sup>+</sup> ions out of the cell and takes two K<sup>+</sup> ions into the cell.<sup>[1,2]</sup>

To understand these functions, a number of one-photon (OP) fluorescent probes have been developed, with SBFI and Sodium Green (SG) being commercially available (Scheme 1).<sup>[3-5]</sup> A disadvantage of these probes is that they are bulky and difficult to load into live cells.<sup>[4c,d,6]</sup> The cell loading could be increased by reducing the molecular weight (MW).<sup>[5]</sup> The second problem, which is common for most OP probes,<sup>[7]</sup> is their short excitation wavelength (below 500 nm). This requirement limits their application in tissue imaging owing to the shallow penetration depth (less than 100 µm), photobleaching, and cellular autofluorescence. An ideal solution to this problem is two-photon microscopy (TPM), which utilizes two near-infrared photons of lower energy for the excitation. Using TPM, intact tissue can be imaged for a long period of time with minimum interference from tissue preparation artifacts that can extend more than 70 µm into the tissue slice.<sup>[8]</sup> However, no two-photon (TP) probe for Na<sup>+</sup> that is capable of imaging [Na<sup>+</sup>]<sub>i</sub> deep inside (more than 100 µm) living tissues has been reported to date.

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Scheme 1. The structures of ANa1, SBFI, and Sodium Green (SG).

To address both of these problems, we designed a TP probe (ANa1) derived from 2-acetyl-6-(dimethylamino)naphthalene (acedan) as the reporter and 1,7-diaza-15-crown-5 as the Na<sup>+</sup> ion receptor (Scheme 1) by considering the following requirements: 1) small MW for cell permeability; 2) high selectivity for Na<sup>+</sup> ions; 3) significant TP cross section for bright TPM images; 4) large spectral shifts in different environments to distinguish between the cytosolic and membrane-bound probes; and 5) high photostability. We adopted acedan from our previous work on various TP probes<sup>[9]</sup> and 1,7-diaza-15-crown-5 from the work of Tsien and Minta.<sup>[3]</sup> Herein, we report that ANa1 is capable of detecting intracellular free Na<sup>+</sup> ions in live cells and tissues at depths greater than 100 µm for long periods of time without mistargeting and photobleaching problems.

ANa1 was prepared in 47 % yield by the coupling reaction between 6-acyl-2-[*N*-methyl-*N*-(carboxymethyl)amino]naphthalene (**A**)<sup>[9]</sup> and *N*-(2-methoxyphenyl)-*N*'-(4-amino-2methoxyphenyl)-1,7-diaza-15-crown-5 (**B**; see the Supporting Information).<sup>[10]</sup> The water solubility of ANa1 was approximately 2  $\mu$ M, which was sufficient to stain the cells (Figure S2, Supporting Information). The fluorescent spectra of ANa1 showed gradual red shifts with the solvent polarity ( $E_T^N$ ) in the



364

order 1,4-dioxane < DMF < EtOH < H<sub>2</sub>O (Figure S1 and Table S1, Supporting Information). The large solvatochromic shifts with increasing solvent polarity indicated the utility of ANa1 as an environment-sensitive probe.

When small increments of Na<sup>+</sup> ions were added to ANa1 in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution (10 mm, pH 7.0) while maintaining the total concentration of Na<sup>+</sup> and K<sup>+</sup> at 135 mM to mimic physiological condition,<sup>[3,5]</sup> OP- and TP-excited fluorescence intensity increased dramatically (Figure 1a, b), probably owing to the blocking of photoinduced electron transfer processes by the complexation of the metal ions. Similar results were observed with  $K^+$ , except that the changes were smaller (Figure S4, Supporting Information). The fluorescence enhancement factor  $[(F-F_{\min})/F_{\min}]$  of ANa1 for OP and TP processes was 8 in the presence of 135 mM Na<sup>+</sup>, a value nearly identical to that of SG (Table S2, Supporting Information).<sup>[5]</sup> Furthermore, Benesi-Hildebrand plots for Na<sup>+</sup> and K<sup>+</sup> binding showed a good linear relationship, indicating 1:1 complexation between probe and cations (Figure S3 and S4, Supporting Information).<sup>[9a]</sup>

The dissociation constants ( $K_d$ ) were calculated from the fluorescence titration curves (Figure S3, Supporting Information). The  $K_d$  value of ANa1 for Na<sup>+</sup> measured in the absence and presence of K<sup>+</sup> ([Na<sup>+</sup>] + [K<sup>+</sup>] = 135 mM) are ( $8.0 \pm 0.5$ ) and ( $20 \pm 1$ ) mM, respectively.<sup>[3]</sup> The values are well within the range of [Na<sup>+</sup>]<sub>i</sub> in the live cells (see above). A similar value

was reported for SG (Table S2, Supporting Information).<sup>[5]</sup> Moreover, the  $K_d$  value of ANa1 for K<sup>+</sup> is (280 ± 15) mM, thus indicating that the selectivity of ANa1 for Na<sup>+</sup> over K<sup>+</sup> is higher than that of SBFI and comparable to that of SG (Table S2, Supporting Information). We also measured the dissociation constant  $(K_d^{i})$  in ionophore-treated astrocyte cells by two-photon spectroscopy (Supporting Information).<sup>[11]</sup> The  $K_d^i$  value of  $(26 \pm 2) \text{ mM}$  is in reasonable agreement with that measured in MOPS buffer (Figure 1b). This value allows quantitative measurement of [Na<sup>+</sup>]<sub>i</sub> by using  $[Na^+]_i = K_d^i[(F - F_{min})/(F_{max} - F)]$ , where  $F_{min}$ ,  $F_{max}$ , and F are the two-photon excited fluorescence (TPEF) intensities in the absence and presence of excess Na<sup>+</sup> and the observed TPEF intensity, respectively.<sup>[12]</sup> Further, the TPEF intensity at a given spot on the ANa1-labeled HeLa cells did not show any decay after continuous irradiation with femtosecond pulses for 60 min, thus indicating its high photostability (Figure S8, Supporting Information).

ANa1 showed a strong response toward Na<sup>+</sup>, much weaker responses toward K<sup>+</sup>, Li<sup>+</sup>, Ca<sup>2+</sup>, and no response toward Mg<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Cu<sup>+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> (Figure 1d). Therefore, this probe can selectively detect [Na<sup>+</sup>]<sub>i</sub> with minimal interference from other biologically relevant cations. Moreover, ANa1 is insensitive to the pH value in the biologically relevant pH range (Figure S5, Supporting Information). The combined results reveal that ANa1 can detect [Na<sup>+</sup>]<sub>i</sub> for a long period of time with

minimum interference from pH value and other metal ions.

The TP action cross section ( $\delta\Phi$ ) of the ANa1-Na<sup>+</sup> complex in buffer solutions indicated a value of 95 GM at 780 nm, which is three- to fivefold larger than those of SG and SBFI (Figure 1c and Table S2, Supporting Information). Moreover, the TPM image of HeLa cells was much brighter when stained with ANa1 than with the commercial probes (Figure S6, Supporting Information), presumably owing to the larger  $\delta\Phi$  value and increased cell loading.

The TPM images of cultured astrocytes labeled with 1  $\mu$ M ANa1 showed very weak TPEF at 360–460 nm and strong TPEF at 500–620 nm (Figure S7, Supporting Information). For comparison, the TPEF spectra from the intense domains in the TPM images of the cells labeled with acedan-derived TP probes for Mg<sup>2+</sup> (AMg1)<sup>[9a]</sup>



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*Figure 1.* a) One-photon fluorescence spectra of 1 μM ANa1 (10 mM MOPS, [Na<sup>+</sup>]+[K<sup>+</sup>]=135 mM, pH 7.0)

in the presence of free Na<sup>+</sup> (0–135 mm). b) Two-photon fluorescence titration of ANa1 with Na<sup>+</sup> in MOPS

buffer ( $\blacksquare$ ) and in astrocytes ( $\square$ ). The excitation wavelength was 780 nm. c) Two-photon action spectra of

of ANa1 in the presence of 200 mm for K<sup>+</sup>, 5 mm for Li<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>; 100  $\mu$ m for Zn<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Cu<sup>+</sup>,

 $Mn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$  (empty bars) and subsequent addition of 100 mm of  $Na^+$  (filled bars).

ANa1 (■), SG (●), and SBFI (▲) in the presence of 135 mM free Na<sup>+</sup>. d) The relative fluorescence intensity

### Communications

and Ca<sup>2+</sup> (ACa1)<sup>[9b]</sup> could be dissected into two Gaussian functions with emission maxima at approximately 440 and 500 nm, respectively, which had been attributed to probes associated with membrane and cytosol. In contrast, the fluorescence spectra of the two domains with different intensities in the ANa1-labeled astrocytes are almost the same (Figure S7b, Supporting Information). Moreover, they are nearly identical to that of ANa1 in the buffer solution with  $\lambda_{max}^{fl} \approx 500$  nm. Hence, ANa1 appears to be predominantly located in the cytosol, probably owing to its low MW. Nevertheless, we have collected TP fluorescence data in the 500–620 nm range to absolutely rule out possible interference from the membrane-bound probes.

To demonstrate the utility of this probe, we monitored TP fluorescence intensity of the ANa1-labeled astrocytes after addition of ouabain, a steroid hormone that inhibits Na<sup>+</sup>/K<sup>+</sup> ATPase and increases the cytosolic free Na<sup>+</sup> concentration.<sup>[11b,13]</sup> The TP fluorescence intensity increased gradually with time and reached a maximum value after 1800 s (Figure 2a–c). The resting [Na<sup>+</sup>], in the astrocytes calculated by  $[Na^+]_i = K_d^i [(F - F_{min})/(F_{max} - F)]$  is (10 ± 2.1) mм. It began to rise after treatment with ouabain (1 mM) and reached a maximum value of  $(50 \pm 5.0)$  mM after 1800 s (Figure 2 c), which is in good agreement with the reported value.<sup>[11b]</sup> We next monitored glutamate-evoked [Na<sup>+</sup>]<sub>i</sub> response of the ANa1-labeled astrocytes. Glutamate is a well-known excitatory neurotransmitter in the brain that is co-transported with three Na<sup>+</sup> ions into the astrocytes, thereby inducing  $[Na^+]_i$ elevation.<sup>[11b,14]</sup> When 100  $\mu$ M glutamate was added, the [Na<sup>+</sup>]<sub>i</sub> began to rise in about 10 s, reached the peak value of (30  $\pm$ 3.2) mM after 60 s, and then decreased to the basal level within 2.5 min, which concurs with literature results.<sup>[11b]</sup> Hence, ANa1 is clearly capable of monitoring the change in [Na<sup>+</sup>]<sub>i</sub> in live cells for a long period time.

To further investigate the utility of this probe in deeptissue imaging, TPM images were obtained from part of a fresh rat hippocampal slice incubated with 20 mM ANa1 for 40 min at 37 °C. The slice of a 14 day old rat was too big to show with one image, so two TPM images were obtained in each plane and combined. The bright-field image of a part of a rat hippocampal slice shows the CA1–CA3 regions as well as the dentate gyrus (DG; Figure 3 a). The TPM images revealed Na<sup>+</sup> distribution in the same region at 100–200  $\mu$ m depth (Figure S9, Supporting Information), but each image represents the distribution exclusively in the given plane. As the structure of the brain tissue is known to be inhomogeneous in its entire depth, we accumulated 25 TPM images at different depths to visualize the distribution of the Na<sup>+</sup> ions (Figure 3b).

The accumulated TPM images show that  $Na^+$  is more abundant in the pyramidal neuron layers of the CA1–CA3 regions than in the DG. The image taken at a higher magnification clearly resolved  $Na^+$  distributions in the pyramidal neuron layer of the CA3 region (Figure 3c). These results demonstrate that ANa1 is capable of detecting the  $[Na^+]_i$  at 100–200 µm depth in live tissue using TPM.

In conclusion, we have developed a TP probe (ANa1) that shows strong TP fluorescence enhancement in response to Na<sup>+</sup> and a dissociation constant  $(K_d^i)$  of  $(26 \pm 2)$  mM in the



**Figure 2.** a,b,d,e) TPM images of 1  $\mu$ M ANa1-labeled astrocytes before (a, 0 s) and after (b, 1800 s) addition of 1 mM ouabain to the imaging solution and before (d, 0 s) and after (e, 260 s) addition of 100  $\mu$ M glutamate to the imaging solution. The TPM images were obtained by collecting the TPEF at 500–620 nm upon excitation at 780 nm with a femtosecond pulse. c,f) The TPEF intensity collected at 500–620 nm as a function of time. Scale bars 30  $\mu$ m. Cells shown are representative images from replicate experiments (n=5).

cell. The probe can be easily loaded into the cells and emits three- to fivefold larger TPEF intensity than SG and SBFI upon complexation with Na<sup>+</sup>. Unlike the previously available probes, this novel probe can selectively detect  $[Na^+]_i$  in live





**Figure 3.** Images of a rat hippocampal slice stained with 20 μM ANa1. a) Bright-field image of the CA1–CA3 regions as well as the dentate gyrus by 10× magnification. b) TPM image by 10× magnification. 25 TPM images were accumulated along the *z* direction at a depth of approximately 100–200 μm. Scale bar 300 μm. c) Magnification at 100× in the pyramidal neuron layer of CA3 regions (red box in Figure 3 b) at a depth of approximately 120 μm. Scale bar 30 μm. The TPM images were collected at 500–620 nm upon excitation at 780 nm with a femtosecond pulse.

cells and living tissues at 100–200  $\mu m$  depth for more than 60 min using TPM.

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