Fluorescent Probes

DOI: 10.1002/anie.201307754

Sensing Acetylcholine and Anticholinesterase Compounds**

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Abstract: *Acetylcholine is a key neurotransmitter, and anticholinesterase agents are essential compounds used as medical drugs, pesticides, and chemical warfare agents. A semisynthetic fluorescence-based probe for the direct, real-time detection of acetylcholine and anticholinesterase compounds is introduced. The probe possesses good sensitivity, tunable detection range, and can be selectively targeted to cell surfaces, thereby making it an attractive tool for applications in analytical chemistry and quantitative biology.*

Acetylcholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline and acetate. It is mainly found at the neuromuscular junction and in the animal synapse, where it terminates the signaling triggered by ACh release.[1] Despite the importance of ACh in biology, no molecular probes exist for its real-time quantification in cell culture experiments or in vivo. Existing tools either permit only indirect detection of ACh or show low signal-to-noise ratio and impact cellular signaling.[2,3] Moreover, a direct quantification of anticholinesterase compounds (ACs), that is, compounds that target AChE such as drugs for AChE-related pathologies,[4] pesticides, and chemical weapons,[5] is of great practical importance. Here we introduce the semisynthetic protein ACh-SNIFIT, a fluorescent probe for the direct, real-time quantification of ACh and ACs within the extracellular matrix. ACh-SNIFIT possesses good signal-to-noise ratio, fast kinetics and can be targeted to selected cells, thereby making it well suited for applications in analytical chemistry and neurobiology.

ACh-SNIFIT is a Förster resonance energy transfer (FRET)-based semisynthetic probe designed according to the SNIFIT concept[6–9] in which the competition between a tethered ligand and free analyte results in a change in FRET efficiency within the probe (Figure 1). Our SNIFIT sensor is constituted of a fusion protein of AChE, CLIP tag, and SNAP tag. Functional ACh-SNIFIT is then formed upon site-specific labeling with synthetic molecules: CLIP is site-specifically labeled with a FRET donor and SNAP with a molecule (Figure 1) containing a FRET acceptor and a ligand for AChE. In the absence of ACs, the tethered ligand is bound to AChE and the two fluorophores are in close proximity. At sufficiently high concentration of ACh or an AC the intramolecular ligand is displaced from AChE, thereby resulting in opening of ACh-SNIFIT and detectable decrease in FRET ratio (Figure 1).

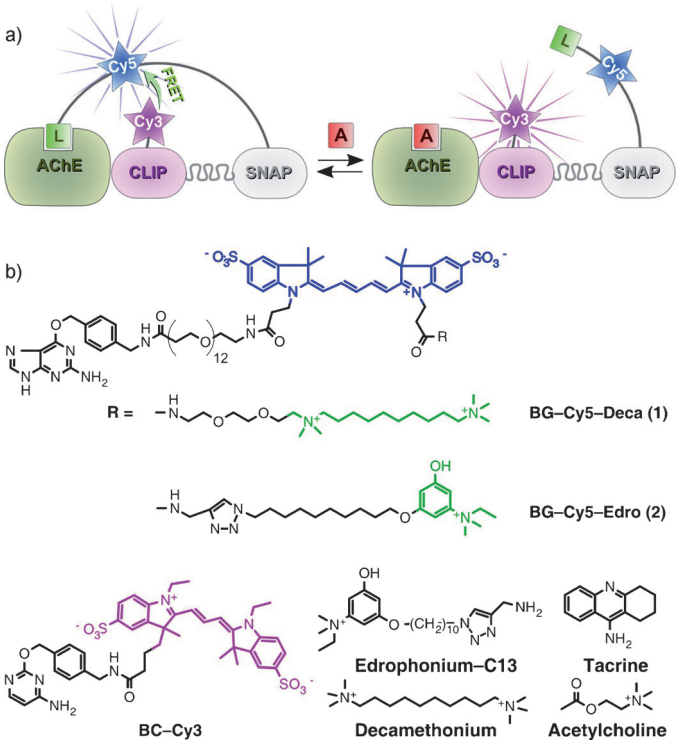

Figure 1. a) ACh-SNIFIT is a fusion protein of AChE, CLIP tag, and SNAP tag in which CLIP is labeled with Cy3 and SNAP with a molecule containing a FRET acceptor and a ligand for AChE. Binding of analyte (A) to AChE displaces the intramolecular ligand (L) and affects the FRET efficiency. b) Molecules utilized in this study: benzylguanine (BG) derivatives **1** and **2** permit the labeling of SNAP fusion proteins; benzylcytosine derivative BC-Cy3 permits the labeling of CLIP.

To maximize the change in FRET efficiency, CLIP and SNAP tags were fused to the C terminus of AChE and thus close to its active site, ensuring proximity of the two fluorophores in the closed state of the probe. The linkers between the three building blocks were optimized (Figure 1) by directly fusing CLIP to the C terminus of AChE and by inserting a long linker between the two tags, ensuring a large distance between the fluorophores in the open state. Active-site residue Ser231 was mutated to Ala to obtain a catalytically inactive probe. The synthetic FRET acceptor was sandwiched between the intramolecular ligand and BG, reacting with SNAP tag (Figure 1).

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[**] This work was supported by the Swiss National Science Foundation. We thank R. Griss, Dr. Reymond, Dr. Lukinavičius, and Dr. Hovius for discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201307754.

We chose as tethered ligand a derivative of decamethonium (Deca, Figure 1), a muscle relaxant drug reported to inhibit AChE with an inhibitory constant of 9.6 μM.[11]

We first characterized the soluble probe by labeling recombinant apo-SNIFIT with BC-Cy3 and BG-Cy5-Deca (Figure 1) to obtain ACh-SNIFIT-D with tethered Deca. Titration of the probe with increasing concentrations of Deca resulted in a decrease in emission intensity of the acceptor fluorophore and increase of the emission intensity of the donor fluorophore (Figure 2), in accordance with the design

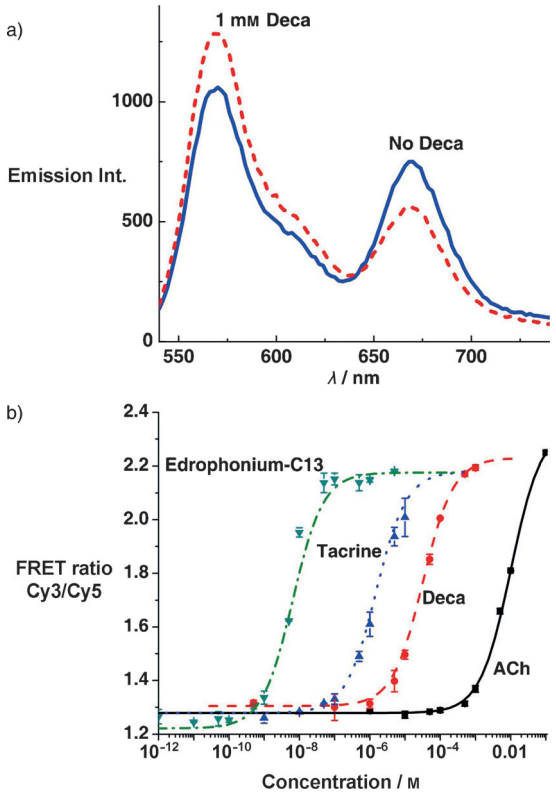

Figure 2. a) Fluorescence emission spectra of ACh-SNIFIT-D in the presence and absence of Deca (λ_{EXC} = 500 nm). b) In vitro titrations of soluble ACh-SNIFIT-D with ACs.

principles. The observed maximum change in FRET ratio was 65%. We then titrated ACh-SNIFIT-D with other ACs and used the c_{50} value, defined as the analyte concentration that resulted in 50% of the maximum ratio change, as a parameter to evaluate the affinity of ACh-SNIFIT for the analyte (Figure 2). The c_{50} values observed for different ACs correlated with the corresponding IC_{50} values measured in enzymatic assays using apo-SNIFIT comprising wild-type AChE (Table 1 and Figure S2 in the Supporting Information). ACh-SNIFIT-D thus also permits a high-throughput-compatible characterization of the relative affinities of ACs.

AChE is a secreted protein and ACh plays its role when released in the synaptic cleft. Thus, we investigated the use of our probe for a direct, noninvasive quantification of concentrations of ACh or ACs on surfaces of living cells. Methods for fluorescence imaging of ACh action on cell surfaces have been demonstrated,[2,3] but they possess several shortcomings.

Table 1: Affinity and inhibitory activity of ACs towards ACh-SNIFIT-D; inhibition assays were performed on apo-SNIFIT with wild-type AChE.

AC	c_{50} [M]	IC_{50} [M]
acetylcholine	$(10.0\pm0.4)\times10^{-3}$	–
decamethonium	$(3.2\pm0.1)\times10^{-5}$	$(8.0\pm0.1)\times10^{-6}$
tacrine	$(1.5\pm0.3)\times10^{-6}$	$(1.1\pm0.04)\times10^{-6}$
edrophonium-C13	$(6.9\pm0.6)\times10^{-9}$	$(5.9\pm0.9)\times10^{-9}$

FRET-based probes utilizing muscarinic acetylcholine receptors have been introduced, but these probes show low ratio changes (< 10%), are saturated at micromolar concentrations of ACh, and require excitation with blue light.[3] Approaches that measure downstream effects such as changes in calcium concentrations only provide an indirect measure of changes in ACh concentrations.[2] We therefore decided to target ACh-SNIFIT to the outer cell membrane of living cells for a direct quantification of ACh and ACs on cell surfaces. We generated doxycycline-inducible cell lines from human embryonic kidney cells (HEK293)[10] expressing the probe protein anchored on the outer cell surface through a growth factor receptor transmembrane domain[6] and labeled cells expressing apo-SNIFIT with BC-Cy3 and BG-Cy5-Deca (Figure 3). Analysis by fluorescence confocal microscopy demonstrated correct labeling and localization of the probe on the cell surface (Figure 3). Since both BC-Cy3 and BG-Cy5-Deca are membrane-impermeable, only those probes properly exported to the cell surface will be labeled.[6]

To demonstrate the use of ACh-SNIFIT for quantification of ACs on cell surfaces we perfused cells displaying ACh-SNIFIT with ACs and analyzed the response of individual cells by plotting the ratio of the fluorescence intensities of the Cy5 and Cy3 channels of a wide-field fluorescence microscope. By perfusing cells with different Deca concentrations we were able to measure the c_{50} value for Deca of ACh-SNIFIT-D on cell surfaces. The measured c_{50} of 48 μM is comparable to the c_{50} value obtained with soluble probe (32 μM; Table 1). We determined an overall FRET-ratio change of 38% (Figure 3). These data demonstrate that ACh-SNIFIT-D permits the direct quantification of ACs on living cells.

To demonstrate that the response range of SNIFITs can be tuned by tethering ligands with different affinity for the receptor protein, we labeled ACh-SNIFIT with the edrophonium derivative BG-Cy5-Edro (Figure 1), resulting in ACh-SNIFIT-E. Edrophonium (Edro) is a reversible cholinesterase inhibitor with a $K_i = 0.62$ μM[11] and the tetherable derivative edrophonium-C13 showed a stronger affinity towards AChE than Deca in vitro (Figure 2 and Table 1). As expected, the measured c_{50} value of ACh-SNIFIT-E for Deca (580 μM) is higher than the corresponding value measured for ACh-SNIFIT-D (48 μM; Figure 3). Thus, ACh-SNIFIT-D responds to Deca concentrations in the range of 5–500 μM, while ACh-SNIFIT-E responds to Deca concentrations in the range of 100–10000 μM, demonstrating how readily the properties of ACh-SNIFIT can be fine-tuned.

For a probe to be applied for live cell imaging a fast response to the analyte concentration is highly desirable; thus we estimated the kinetics of the opening and closing of ACh-

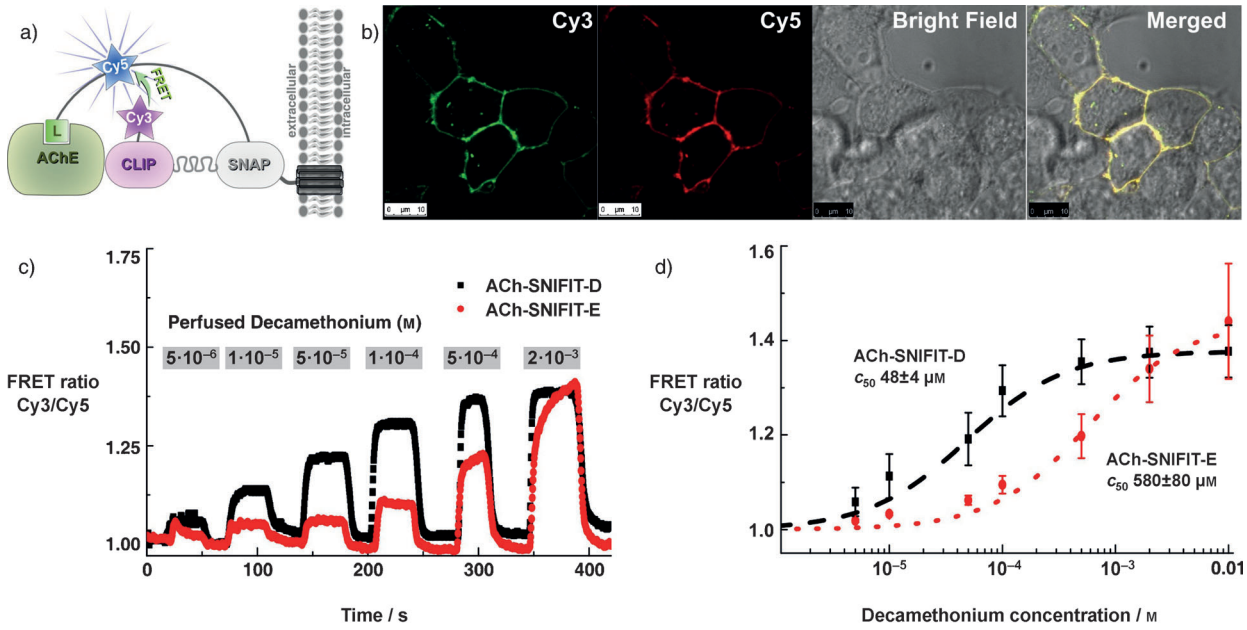

Figure 3. a) Anchored ACh-SNIFIT model. b) Confocal images of HEK cells expressing ACh-SNIFIT and labeled with BC-Cy3 and BG-Cy5-Deca. Scale bars: 10 μm. c) Perfusions of decamethonium on cells expressing ACh-SNIFIT and d) quantification of sensor response. ACh-SNIFIT-E (red ●) with the stronger tethered ligand edrophonium responds at higher analyte concentrations than ACh-SNIFIT-D (black ■).

SNIFIT on the cell surface. Opening of the SNIFIT probe is mainly governed by the k_{off} of the tethered ligand from the receptor protein, while the closing of the probe is related to the k_{off} of the analyte.[7] We perfused Deca and tacrine (Tac), a cholinesterase inhibitor previously used to treat Alzheimer's disease[4] (in vitro $c_{50} = 1.5$ μM), on live cells expressing ACh-SNIFIT-D. The probe readily responded to the addition of the inhibitors with opening half-life (t_{open}), which is defined as the time to reach half-maximum ratio change, for the two analytes of 1.5 s and 1.8 s, respectively (Table 2). Note that these rates are similar to the speed with which the bath solution inside our perfusion chamber is exchanged;[7] the real probe opening could thus be faster. The closing half-life (t_{close}) was measured by washing out the inhibitor. Deca showed a t_{close} around 8-fold smaller than tacrine (5 s and 39 s, respectively). This suggests a smaller k_{off} value of tacrine compared to Deca, a result compatible with the lower c_{50} value measured for tacrine in vitro (Table 1). To demonstrate that opening and closing kinetics can be controlled by using different tethered inhibitors, we perfused Deca on cell-anchored ACh-SNIFIT-E. As expected, using a stronger tethered ligand increased t_{open}, while t_{close} values remained unchanged (Table 2). These experiments demonstrate that ACh-SNIFIT responds on a second timescale and that it can be used to determine the kinetics of the interaction of ACs with AChE.

Table 2: Kinetics of opening and closing of ACh-SNIFIT with different tethered ligand and analytes perfused.

Perfused	ACh-SNIFIT-D Deca (100 μM)	ACh-SNIFIT-D Tac (30 μM)	ACh-SNIFIT-E Deca (100 μM)	ACh-SNIFIT^{WA}-E ACh (50 mM)
t_{open}	(1.5±0.2) s	(1.8±0.2) s	(3.6±0.3) s	(2.4±0.4) s
t_{close}	(5±1) s	(39±3) s	(4±1) s	(4±1) s

Finally, we investigated the use of our probe for visualizing ACh concentrations on cell surfaces. Initial experiments demonstrated that when perfusing cells with concentrations of ACh as high as 100 mM, ACh-SNIFIT-E gave more reproducible results than ACh-SNIFIT-D (data not shown). However, even at 100 mM ACh we observed only a partial opening of the sensor (Figure 4). To increase the sensitivity of ACh-SNIFIT-E towards ACh we mutated Trp310 to Ala, as this residue has been reported to specifically contribute to the interaction with edrophonium derivatives.[12] The resulting probe ACh-SNIFIT^{WA}-E responded in perfusion experiments to ACh in the range 1–100 mM with c_{50} of 20 mM and 52% FRET-ratio change (Figure 4). The ACh concentration reached upon presynaptic release in the synaptic cleft is not precisely known, but in cholinergic synaptic vesicles is estimated to be 100 mM,[13] thus above the c_{50} value of ACh-SNIFIT^{WA}-E. The kinetics of sensor opening and closing (Table 2) most likely is too slow to follow concentration changes during synaptic release. However, nicotinic ACh receptors in the central nervous system are extrasynaptic, far from the region of ACh release, and ACh action is significantly slower than the kinetics of synaptic release.[13] We have demonstrated that the c_{50} value of ACh-SNIFIT can be easily fine-tuned and we believe this probe represents a good starting point for imaging of physiological relevant concentration changes of ACh. Furthermore, FRET sensors with comparable ratio changes have already been shown to be suitable for applications in vivo.[2]

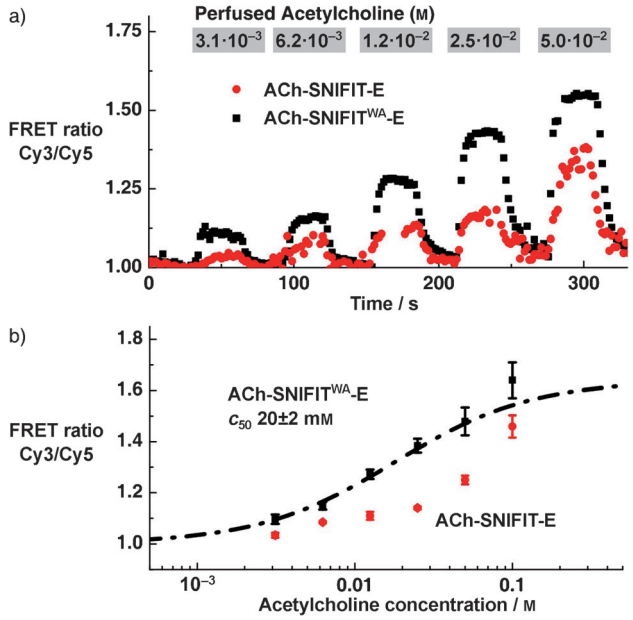

Figure 4. Titrations with ACh of ACh-SNIFIT-E cells: a) perfusions and b) concentration dependency. ACh-SNIFIT^{WA}-E was engineered introducing the W310A mutation to respond to low millimolar concentrations of ACh.

In summary, we developed a robust and versatile real-time fluorescent probe for acetylcholine and anticholinesterase compounds. ACh-SNIFIT proved to be well-performing both in homogenous assays and when anchored to the outer cell surface of living cells. The ratiometric response to ACs makes this probe an attractive tool for quantification of drugs, pesticides, nerve agents, or their antidotes as well as for imaging of biodistribution of ACs. SNAP-tag expression and labeling in living animals[14] and imaging of implanted fluorescent cell lines[2] have been recently demonstrated, and we envision the future use of ACh-SNIFIT either by direct expression by the host cell or implantation of ACh-SNIFIT cell lines.

Experimental Section

Detailed experimental procedures for the synthesis of BG-Cy5-Deca and BG-Cy5-Edro, for protein expression and characterization, and for the biochemical experiments are given in the Supporting Information. apo-ACh-SNIFIT, as a soluble protein or anchored to the outer cell membrane, has been cloned using standard cloning techniques and transfected in HEK293 cells. Cells were grown at 37 °C in DMEM (Gibco) complemented with 10 % fetal bovine serum (Gibco). Labeling reactions, in vitro titrations, and perfusion experiments were performed in HBSS (Gibco) at 25 °C.

Received: September 3, 2013
Revised: October 24, 2013
Published online: December 13, 2013

Keywords: acetylcholine · acetylcholinesterase · biosensors · FRET · imaging

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