Single-Molecule Imaging of a Fluorescent Unnatural Amino Acid Incorporated Into Nicotinic Receptors

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ABSTRACT We report on the first, to our knowledge, successful detection of a fluorescent unnatural amino acid (fUAA), Lys(BODIPYFL), incorporated into a membrane protein (the muscle nicotinic acetylcholine receptor, nAChR) in a living cell. Xenopus oocytes were injected with a frameshift-suppressor tRNA, amino-acylated with Lys(BODIPYFL) and nAChR (α/β19'GGGU/γ/δ) mRNAs. We measured fluorescence from oocytes expressing nAChR β19'Lys(BODIPYFL), using time-resolved total internal reflection fluorescence microscopy. Under conditions of relatively low receptor density (<0.1 receptors/μm²), we observed puncta with diffraction-limited profiles that were consistent with the point-spread function of our microscope. Furthermore, diffraction-limited puncta displayed step decreases in fluorescence intensity, consistent with single-molecule photobleaching. The puncta densities agreed with macroscopic ACh-induced current densities, showing that the fUAA was incorporated, and that receptors were functional. Dose-response relations for the nAChR β19'Lys(BODIPYFL) receptors were similar to those for wild-type receptors. We also studied nAChR β19'Lys(BODIPYFL) receptors labeled with α-bungarotoxin monoconjugated with Alexa488 (αBtxAlexa488). The nAChR has two αBtx binding sites, and puncta containing the Lys(BODIPYFL) labeled with αBtxAlexa488 yielded the expected three discrete photobleaching steps. We also performed positive control experiments with a nAChR containing enhanced green fluorescent protein in the γ-subunit M3-M4 loop, which confirmed our nAChR β19'Lys(BODIPYFL) measurements. Thus, we report on the cell-based single-molecule detection of nAChR β19'Lys(BODIPYFL).

INTRODUCTION

Fluorescent labeling of proteins is a valuable technique for understanding biological processes at the cellular and subcellular levels. In a widely used method with subcellular resolution, a fluorescent protein (FP) (Fig. 1 A) (1) is genetically fused to a protein of interest (2,3). Such FP fusions are used to measure protein expression levels, colocalization, and trafficking. The FP moieties, however, are typically ~27 kDa, and therefore may interfere with protein function, folding, and stability. Several methods were developed to overcome these limitations. For example, fluorophores with appropriate chemical groups can react with appropriate amino-acid side chains, but such fluorophores label all proteins in a nonselective manner in living cells (4,5). The selective posttranslational labeling of proteins was achieved by introducing unnatural amino acids that can then be derivatized via bio-orthogonal reactions (6–8). However, these reactions may not reach completion, and the reagents may partition nonspecifically into membranes. Orthogonal enzymatic labeling approaches have expanded the toolkit for protein labeling, but require the insertion of a peptide sequence and subsequent labeling (9,10). Peptide sequences with binding affinity or reactivity toward lanthanide ions (11,12) and synthetic dyes (13) can also be encoded into proteins and synthetic peptides. Nevertheless, the intracellular labeling of proteins remains challenging.

Fluorescent unnatural amino acids (fUAAs) represent an attractive alternative strategy. Previously, fUAAs were incorporated into various types of proteins, using nonsense (14–19) and frameshift (15,18) suppression methodologies. Orthogonal tRNA/aa-tRNA synthetase pairs that can incorporate fUAAs were also developed (20–22). Several fUAAs were incorporated into functional proteins and detected with conventional cuvette or gel-based fluorescence measurements. Fluorescence moieties that were incorporated include: 5-hydroxytryptophan (21), 7-azatryptophan (16), dansyl (20), coumarin (23), NBD-Dap (14), Aladan (19), and BODIPYFL derivatives (15,18). These fluorophores span a wide spectral range, indicating the generality of nonsense and frameshift-suppression methodologies for the site-specific incorporation of fUAAs into proteins.

For example, fUAAs were previously incorporated into receptors and ion channels heterologously expressed in Xenopus oocytes by nonsense suppression. The NBD-Dap and Aladan were incorporated into the functional neurokinin-2 (NK2) receptor (14) and voltage-gated potassium channel Kir2.1 (19), respectively. Plasma membranes from oocytes were isolated, and the fluorescence of NBD-Dap incorporated into the NK2 receptor was measured with cuvette fluorescence spectrometry. In addition, a NK2 cysteine mutant was labeled with a tetramethylrhodamine (TMR) thiol-reactive fluorescent probe, and Förster (or fluorescence) resonance energy transfer (FRET) was detected between the NBD donor and TMR (14). However, to the best of our knowledge, there are no reports of fluorescence imaging with fUAAs incorporated into membrane proteins in living cells. Live-cell fluorescence imaging is essential in understanding the fundamental...
function and trafficking of ion-channel proteins. This is especially true of neuronal ion-channel proteins, whose function (24) and expression patterns (25) have subcellular specificity.

To extend fUAAs incorporation to living cells and also to the single-molecule level, we chose an ion-channel molecule that is well-characterized, but still poses important scientific questions. We studied the incorporation of Lys(BODIPYFL) (Fig. 1B) into the mouse muscle nicotinic acetylcholine receptor (nAChR) heterologously expressed in *Xenopus* oocytes. The nAChRs are cation-selective, ligand-gated ion channels (LGICs) in the pentameric Cys-loop superfamily.

We studied the photobleaching of diffraction-limited puncta to assess the incorporation of fUAAs. We also compared fluorescence signals from the nAChR β19’Lys(BODIPYFL) labeled with a second fluorophore, α-bungarotoxin mono-conjugated with Alexa488 (αBtxAlexa488). We compared signals from nAChR containing enhanced green fluorescent protein in the γ-subunit M3–M4 loop (γeGFP) with our nAChR β19’Lys(BODIPYFL) observations. Fluorescence measurements with nAChR β19’C labeled with BODIPYFL-C3-MTS (Fig. 1E), a thiol-reactive BODIPYFL variant, were not possible because BODIPYFL-C3-MTS does not label the β19’C residue. Thus, our study illustrates the advantages and current limitations of biosynthetically incorporating fUAAs into ion channels at buried residues.

**METHODS**

**Reagents and materials**

**Preparation of α-NVOCLys(BODIPYFL)-O-dCA**

All reagents were purchased from Sigma-Aldrich (St. Louis, Mo.) unless otherwise noted. The high-performance liquid chromatography (HPLC) instrumentation was described elsewhere (35). We prepared α-NVOCLys(BODIPYFL)-OCH2CN using the protocol reported for α-NVOCl-Lys(C3-MTS) (31). Briefly, **L-Lys (100 mg, 0.41 mmol);** Bachem, Torrance, CA), **NVOCl-C1 (168 mg, 0.62 mmol),** and **Na2CO3 (65 mg, 0.62 mmol)** were dissolved in 18 M2 H2O/dioxane (1:1, 45 mL) and stirred at room temperature for 8 h. The presence of product was confirmed with APCI-MS: calculated for **C21H30N3O10S2 484.19;** found [M – H]+: 484.1. Then, 15 mL of 1 M NaHCO3 was added to the reaction, and α-NVOCl-Lys(BODIPYFL) was extracted with CH2Cl2 (30 mL × 3). The CH2Cl2 fractions were combined and removed by rotary evaporation. The sample was not further purified, and **α-NVOCl-Lys(BODIPYFL) (~19.8 mg, 0.04 mmol)** was dissolved in CH2Cl2 (3 mL). The TFA (3 mL) was added, and the mixture was stirred at room temperature. The CH2Cl2 was added 30 min later, and the solvents were removed with rotary evaporation. The **α-NVOCLys(BODIPYFL)** was purified with semipreparative HPLC, using a linear gradient of 100% 25 mM NH4Ac buffer (pH 4.5) against the organic phase (98%/2% ACN/M2 H2O) for ~60 min. Fractions corresponding to the 350-nm absorption peak from the NVOC group were pooled and lyophilized. The NVOC absorption extinction coefficient at 260 nm ε260 0.140 M−1 cm−1 was used.
to determine that 12.1 mg (77% yield) of product were obtained. The presence of α-NVOC-L-Lys was confirmed by positive mode atmospheric pressure chemical ionization mass spectrometry: calculated for C_{32}H_{38}BF_{2}N_{6}O_{9}^+ 386.16; found [M + H]^+: 386.1. Twelve milligrams of α-NVOC-L-Lys and 5 mg of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY FL, SE, Invitrogen, Carlsbad, CA), were dissolved in 2 mL of dimethylformamide (DMF) and 4.4 μL of diisopropylethylamine, and mixed for 7 h. An aqueous workup was performed with 25 mL of 1 M NaHSO₄, and the product was extracted with CH₂Cl₂ (30 mL × 3). The CH₂Cl₂ extractions were pooled together and dried over Na₂SO₄ for 1 h. The CH₂Cl₂ was removed with rotary evaporation. The DMF was removed by simultaneously immersing the round-bottomed flask with the sample in a 60 °C water bath and attaching it to a high vacuum line with solvent traps. Next, the sample was mixed with 42 μL of CICH₂CN, 3 μL of triethylamine, and 1 mL of DMF. The reaction was allowed to proceed for ~7 h before an aqueous workup was performed with 15 mL of NaHCO₃ and CH₂Cl₂ (25 mL × 3). The organic phase was dried over Na₂SO₄ for 30 min, and the CH₂Cl₂ was removed with rotary evaporation. The product was purified with semipreparative HPLC, using a linear gradient of 100% 25 mM NH₄Ac buffer (pH 4.5) against the organic phase (98%/2% ACN/18 mM H₂O) for ~60 min. The fractions that had both absorbance peaks of the NVOC (~350 nm) and BODIPYFL (~504 nm) and identical elution times were collected, pooled, and lyophilized. The product yield was 6.8 mg (76% yield). The sample was identified by positive mode electrospray ionization mass spectrometry: calculated for C_{32}H_{38}BF_{2}N_{6}O_{9}^+ 699.28; found [M + H]^+: 699.2, and the solvent was lyophilized. The 1H NMR (300 MHz, CDCl₃) δ 7.70 (s, 1H), 7.36 (s, 1H), 7.13 (s, 1H), 7.00 (d, 1H), 6.44 (m, 2H), 6.31 (d, 1H), 6.23 (s, 1H), 5.43 (m, 2H), 4.82 (s, 2H), 4.24 (m, 1H), 3.95 (s, 3H), 3.89 (s, 3H), 3.15 (m, 4H), 2.53 (m, 2H), 2.27 (s, 3H), 2.17 (m, 3H), 1.8 (m, 2H), and 1.42 (m, 4H).

Preparation of BODIPYFL-C₃-MTS

Twelve milligrams of 3-aminopropyl methanethiosulfonate hydrobromide (Toronto Research Chemicals, Toronto, Ontario, Canada) and 250 μg of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY FL, SE, Invitrogen), were dissolved in 1 mL of DMF and stirred under argon (Ar) for ~24 h (in vacuo). The presence of sample was confirmed with analytical HPLC. Preparative HPLC was used to purify α-NVOC-L-Lys(BODIPYFL)-O-BCA, and negative mode electrospray ionization mass spectrometry: calculated for C_{36}H_{42}BF_{2}N_{6}O_{9}P_{2}^− 527.35; found [M – H]^−: 527.3. The product was purified with semipreparative HPLC, using a linear gradient of 100% 25 mM NH₄Ac buffer (pH 4.5) against the organic phase (98%/2% ACN/18 mM H₂O) for ~60 min. The fractions that had both absorbance peaks of the NVOC (~350 nm) and BODIPYFL (~504 nm) and identical elution times were collected, pooled, and lyophilized. The product yield was 8.6 mg (66% yield), based on matrix assisted laser desorption ionization mass spectrometry at 260 nm and matrix assisted laser desorption ionization mass spectrometry. The major peak indicated that YFaFSACCγ-α-NVOC-L-Lys(BODIPYFL) was the principal product of the ligation reaction.

**Construct preparation and mRNA transcription**

The mouse muscle nAChR subunits (α, γ, and δ) are in the pAMV vector. The β19/GGGu construct was prepared by QuikChange (Stratagene, La Jolla, CA) mutagenesis on the masked β-subunit in the pAMV vector (37). The mutation was verified by sequencing (California Institute of Technology Sequecing/Structure Analysis Facility). The mRNAs were prepared from NotI linearized plasmid DNA, using a T7 message Machine kit (Ambion), and were purified with the RNAse Mini Kit (Qiagen, Valencia, CA). Concentration was determined by absorption at 260 nm.

The γeGFP construction was prepared as follows. The eGFP insertion in the γ-M3-M4 cytoplasmic domain has no effect on the electrophysiological properties of nicotinic receptors (αβγeGFP) (41). Therefore, we inserted eGFP after position S380 in the γ-M3-M4 cytoplasmic loop by polymerase chain reaction. The construct was synthesized from three segments by polymerase chain reaction. Primers to amplify the first segment are γeGFP-Forward 1 (ACC ATG GCC CAA GGG GGC CAG AGA CCT CAT CTC CTT GTC CGT, where ATG is the initiation codon of the γ-subunit); γeGFP-Reverse No. 1 (5′- CAG CTC CTC GCC CTT GCT CAC CAT TGA GGA AGA ACC ATT CTG TCG-3′; bold sequences represent the N-terminal of eGFP). The second segment was synthesized by γeGFP-Forward 2 (5′- CGA CTC CAG AAT GGC TCT TCC TCA ATC GTG AGC AAG GGC GAG GAG CAG CTG-3′; bold sequences represent the N-terminal of the eGFP) and γeGFP-Reverse No. 2 (5′- CTC TCG AGC CAT GAT GGG CCA CCC CTT GTA GCA CTC GAT GCC GAG GAG GAC CAG GGT GGC GAG GCT TCC-3′; bold sequences represent the C-terminus of eGFP). The third segment was synthesized by the following γeGFP-Forward No. 3 (5′- CTC GCC ATG GAG CAG GCT TAC AAG GGG TGG CCC ATC ATG GCT CGA GAG-3′; bold sequences represent the C-terminus of the eGFP) and γeGFP-Reverse No. 3 (5′- GTC GAC TCA TGG TGA CAA CAG GAT GAT GGG GGG GGC GAG GCT TCC-3′; bold sequences represent the termination codon of the γ-subunit). Finally, the entire construct was amplified from all three segments, using γeGFP-Forward No. 1 and γeGFP-Reverse No. 3, and was subcloned into the pAMV vector. The entire construct was verified by DNA sequencing (Davis Sequencing, Davis, CA).

**Oocyte preparation, injection, and αBtxAlexa488 labeling**

Stage VI oocytes from Xenopus laevis were isolated and maintained at 16 °C in ND96 solution consisting of 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES (pH 7.5), supplemented with Na pyruvate (2.5 mM), gentamycin (50 μg/mL), theophylline (0.6 mM), and horse serum (5%). Before injection, YFaFSACCγ-α-NVOC-L-Lys(BODIPYFL) was irradiated at 350 nm to cleave the NVOC group, yielding the deprotected RNA-fUA (YFaFSACCγ-α-NVOC-L-Lys(BODIPYFL)). Then the YFaFSACCγ-α-NVOC-L-Lys(BODIPYFL) was kept at 4 °C until mixing with the mRNA and loaded for oocyte injection. Each oocyte was injected with a 1:1 volume mixture of YFaFSACCγ-α-NVOC-L-Lys(BODIPYFL) (12.5 or 25 ng) and mRNA (25 ng of total concentration ratio of 2.5:1:1 for αβ19/GGGu/γδ subunits) in a 50-nL volume. Wild-type (WT) nAChR α, β, and δ and either WT γ or γeGFP mRNA were mixed at a ratio of 2:1:1:1 and diluted to a final concentration of 1 ng/25 nL. Oocytes were labeled with α-bungarotoxin conjugated to Alexa Fluor 488 (αBtxAlexa488) (Invitrogen), dissolved in ND96. We experimented with various labeling conditions and incubation times, and determined that incubating oocytes with 20–40 nM αBtxAlexa488 for 8–12 h resulted in optimal labeling. The incubations were terminated by transferring oocytes to a 5 mg/mL bovine serum albumin solution, followed by two ~30-min washes in ND96 before imaging. The manufacturer furnished αBtxAlexa488 at a nominal concentration of 1 μg/mL. We determined the molar ratio of αBtx...
to Alexa Fluor 488 more precisely, using the known αBtxAlexa488 MW = 8600 (αBtx MW = 7966.23 Da, Alexa Fluor 488 carboxylic acid = 532.5 Da, and linker MW = unknown, assumed to be 100 Da), the Alexa488 absorption peak at 488 nm, the extinction coefficient of 65.000 M⁻¹ cm⁻¹ (36), and the NanoDrop (Thermo Scientific, Wilmington, DE) A₂₈₀ protein assay. The molar ratio of αBtxAlexa488 was 1.0:74. The attempted labeling of the nAcHR β19C receptor with 5 nM and 5 µM BODIPYFL-C₃-MTS was performed as previously reported (30).

Electrophysiology

A two-electrode voltage clamp was used to record currents from oocytes. Recordings were performed 24–48 h after injection with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA). The pipette microelectrodes were filled with 3 M KCl and had resistances ranging from 0.5–2 MΩ. Oocytes were perfused continuously with a calcium-free solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES (pH 7.5). The ACh-induced currents were recorded in response to a series of ACh applications (1–1000 µM).

Total internal reflection fluorescence microscopy

Xenopus oocytes were prepared for single-molecule TIRF microscopy as follows. The oocytes were transferred to a petri dish filled with hypertonic solution (220 mM Na aspartate, 10 mM EDTA, 2 mM MgCl₂, and 10 mM HEPES, pH 7.4) and incubated for 5–10 min. The vitelline membrane was removed with forceps, under a dissecting microscope. Oocytes were transfected into an imaging chamber mounted on the microscope stage. The imaging chamber bottom had a glass coverslip that was cleaned with one of two solutions (220 mM Na aspartate, 10 mM EDTA, 2 mM MgCl₂, and 5 mM HEPES (pH 7.5). The ACh-induced currents were recorded in response to a series of ACh applications (1–1000 µM).

Image analysis

The images acquired with Slidebook were exported as 16-bit time series stack files in TIFF format. The ImageJ (National Institutes of Health) region of interest (ROI) manager was used to document the puncta in the first frame of time stacks. Rectangular 15 × 15 pixel ROIs, centered on a putative single punctum, were duplicated and batch-processed using Matlab R2006a. The point-spread function (PSF) of individual puncta were fit to a two-dimensional Gaussian function:

\[
\text{PSF}(x, y, x_0, y_0, S_x, S_y, A, B) = B + A \exp \left\{ -\frac{(x - x_0)^2}{2S_x^2} - \frac{(y - y_0)^2}{2S_y^2} \right\},
\]

where \((x_0, y_0)\) is the centroid of the punctum, \(S_x\) and \(S_y\) are the respective standard deviations along the x and y axes, A is the amplitude, and B is the baseline. Next, the full width at half-maximum (FWHM\(_x\)) along the x and y axes were determined, FWHM\(_x\) = 2 × \(\sqrt{\ln(4)}\) × \(S_x\), and compared against the FWHM\(_{theoretical}\) PSF of the microscope, which is approximated by (43,44):

\[
\text{FWHM}_{\text{theoretical}} = \frac{0.55 \times \lambda_{\text{emission}}}{NA},
\]

where \(\lambda_{\text{emission}}\) is the emission peak of the fluorescent molecule, and NA is the numerical aperture of the objective (NA = 1.45). The PSF is deformed by several factors, including spherical aberrations, excitation-field nonuniformity (45,46), pixel nonuniformity, background nonuniformity (45), and others. Demuro and Parker reported that muscle nAChRs undergo lateral motions of ±<50 nm on a time scale of ~10 s (47). Therefore, puncta with FWHM\(_{x,y}\) = 195 ± 74.5 nm (or 2.91 ± 1 pixels) were classified as diffraction-limited, immobile spots, and were selected for puncta density and photobleaching analysis. Time-lapse images with minimal lateral and vertical drift were selected for photobleaching step analysis. The peak pixel value against time was plotted for ROIs with puncta that fulfilled the stated FWHM\(_{x,y}\) criteria. Time-lapse traces were imported into Clampfit and analyzed.

RESULTS

In the nonsense and frameshift-suppression methodologies for site-specific UAA incorporation, the chemically aminoacylated tRNA is a stoichiometric reagent that limits the number of functional channels that can be expressed (48). This low expression level is well-suited to the sensitivity of electrophysiology. Here we demonstrate that single-molecule TIRF microscopy complements the sensitivity of electrophysiology. TIRF microscopy excites only molecules that are within ~100 nm of the glass surface, thereby minimizing interference from cytoplasmic autofluorescence, including unincorporated fUAAs (tRNA-fUAA or free fUAA). Thus, TIRF microscopy, integrated with a CCD camera with high expression level is well-suited to the sensitivity of electrophysiology.

IRNA-fUAA and heterologous expression in Xenopus oocytes

Based on previous cell-free synthesis applications (15,49,50) and photophysical properties, i.e., favorable absorption coefficient (ε = 80,000–91,000 M⁻¹ cm⁻¹) and quantum yield
approaching 1.0 (15,36), Lys(BODIPYFL) (Fig. 1 B) was selected as the fUA for incorporation into nAChR. The single amino-acid substitution of Lys(BODIPYFL) (Fig. 1 B) is presumably less perturbing than substituting eGFP (Fig. 1 A) into the transmembrane α-helix (Fig. 1 D). For fUA incorporation, we chose to use the yeast Phe frameshift-suppressor (YFaFS_{ACCY}) that recognizes the quadruplet codon GGGU and contains seven mutations in the acceptor stem (denoted by lower-case “a”) to avoid recognition by endogenous aminoacyl-tRNA synthetases (aaRSs). The YFaFS_{ACCY} is recognized least by the endogenous aaRSs of any suppressor tRNA screened in our laboratories, and therefore should not incorporate natural amino acids at the suppression site (37,51,52). As described in Methods, we first synthesized dCA-Lys(BODIPYFL), the fUA chemically amino-acylated on the dinucleotide deoxyCA, and then enzymatically ligated dCA-Lys(BODIPYFL) to the frameshift-suppressor tRNA lacking the terminal CA nucleotides (74mer-YFaFS_{ACCY}), which yielded YFaFS_{ACCY}-Lys(BODIPYFL), the full-length 76mer-tRNA ligated to the fUA. The YFaFS_{ACCY}-Lys(BODIPYFL) was mixed with nAChR α/β19/1GGGU/γβ mRNA and injected into Xenopus oocytes. After incubation for 1–2 days, the presence of nAChR β19’Lys(BODIPYFL) on the plasma membrane was confirmed with two-electrode voltage-clamp electrophysiology (currents for a series of 5-s ACh applications are shown in Fig. 2 A). The average maximal currents evoked by 1 mM ACh (\(I_{\text{max}}\)) were 63 ± 0.6 μA (\(n = 5\) oocytes) and 3.5 ± 0.2 μA (\(n = 4\) oocytes) for oocytes injected with 25 and 12.5 ng YFaFS_{ACCY}-Lys(BODIPYFL), respectively. The ACh dose-response relationships were generated for nAChR β19’Lys(BODIPYFL) and WT nAChR. The measured EC_{50} values were 38 ± 2 and 60 ± 4 μM ACh, respectively, and the Hill coefficients were 1.1 ± 0.05 and 1.3 ± 0.01, respectively (\(n = 5\) and 9 oocytes, respectively) (Fig. 2 B). Substantially smaller currents were detected from oocytes injected with nAChR β19’GGGU mRNA only (\(I_{\text{max}} = 0.78 ± 0.5\) μA; \(n = 3\) oocytes) or nAChR β19’GGGU mRNA with 74mer-YFaFS_{ACCY} (\(I_{\text{max}} = 1.1 ± 0.3\) μA; \(n = 3\) oocytes) (Fig. 2 C).

**Single-molecule TIRF microscopy of an incorporated fUA**

Single-molecule TIRF microscopy was used to detect the presence of nAChR β19’Lys(BODIPYFL) in the plasma membrane (Fig. 3, A and B). Sparse fluorescent puncta (<0.10 puncta/μm²) were detected 24 h after coinjection of YFaFS_{ACCY}-Lys(BODIPYFL) (12.5 ng/oocyte) with nAChR β19’GGGU mRNA (25 ng/oocyte; \(n = 5\) oocytes; Fig. 3 A and Fig. S1 A in the Supplementary Material). Control oocytes from the same batch were always injected with YFaFS_{ACCY}-Lys(BODIPYFL), (i.e., with tRNA-fUA, but no mRNA), and sparse puncta were detected at lower levels (< 0.015 puncta/μm²) (Fig. S1 B). The PSF of emission profiles (Fig. 3 B) of fluorescent single molecules were fitted to a two-dimensional Gaussian function (45,53) (Fig. 3 C). We compared the two-dimensional Gaussian FWHM_x and FWHM_y, along the x and y axes against the theoretical FWHM. The emission peak of BODIPYFL is ∼515 nm (36). Therefore, we defined puncta with a FWHM_{x,y} = 195 ± 74.5 nm (1 pixel) as diffusion-limited spots for further analysis. Twenty percent to 50% of all puncta detected were diffusion-limited regions, consistent with the PSF of the TIRF microscope. Time-series traces with single-molecule photobleaching events were observed from puncta with diffusion-limited regions (Fig. 3 D). Time-series imaging experiments demonstrated that the puncta originated from one (80%), two (17%), or three (3%) molecules (\(n = 3\) oocytes, 151 puncta), as determined by discrete photobleaching events. Thus, although the majority of puncta represented a single molecule, a subset represented more than one molecule per diffusion-limited spot.

To verify our observations with nAChR β19’Lys (BODIPYFL), we synthesized a thiol-reactive BODIPYFL derivative, BODIPYFL-C3-MTS (Fig. 1 C), and previously labeled with sulforhodamine-MTS (MTSR), and voltage-clamp fluorometry was performed (30). Thus, we experimented with labeling nAChR β19’C with BODIPYFL-C3-MTS, to compare the two labeling
strategies directly. However, the TIRF microscopy images show no difference between uninjected oocytes and those expressing nAChR β19'C labeled with BODIPYFL-C3-MTS. This observation agrees with reports that nAChR β19'Ci is primarily accessible to positively charged thiol-reactive probes (54).

Ulbrich and Isacoff demonstrated the power of counting ion-channel subunits by using genetically fused fluorescent proteins (55). Therefore, we evaluated an alternative strategy, which consisted of generating a nAChR mutant with genetically fused eGFP. Previous data indicated that function and trafficking are well-preserved when FPs are incorporated into the M3-M4 intracellular loop of muscle (and neuronal α4β2) nicotinic receptors (41,56,57). Thus, a nAChR γ-subunit construct with eGFP inserted in the M3-M4 loop (nAChR γeGFP) served as a positive control for our observations with nAChR β19'Lys(BODIPYFL). The nAChR γeGFP receptors were heterologously expressed in oocytes at similar levels as in nAChR β19'Lys(BODIPYFL), and diffraction-limited puncta were detected (Fig. 4 A and Fig. S1 C). Puncta from uninjected oocytes were essentially nonexistent (Fig. S1 D). The nAChR γeGFP puncta consisted of ~1 (80%) (Fig. 4 B) and 2 (20%) photobleaching steps (n = 3 oocytes, 179 puncta), i.e., similar to nAChR β19'Lys(BODIPYFL). The amplitudes obtained from the Gaussian fits of individual puncta from nAChR β19'Lys(BODIPYFL) and nAChR γeGFP at a similar excitation power density (133 mW/cm²) were compared. The nAChR β19'Lys(BODIPYFL) and nAChR γeGFP displayed log-normal distributions (58), with a peak value at 1064 counts and log standard deviation (log SD) of 0.43 (n = 3 oocytes, 135 puncta), and a peak value at 501 counts with a log SD of 0.51 (n = 2 oocytes, 78 puncta), respectively (Fig. 5).

nAChR fluorescent ligand confirms the presence of nAChR β19'Lys(BODIPYFL) and nAChR γeGFP

To gain confidence that the puncta originated from nAChR β19'Lys(BODIPYFL) and nAChR γeGFP receptors, we...
compared the signals with those for receptors labeled with α-bungarotoxin monoconjugated to Alexa488 (αBtxAlexa488). Two αBtx molecules bind each muscle nAChR irreversibly (59) on the time scale of interest in this study. Thus, time-series traces with three photobleaching events were expected when we labeled nAChRb19LyS(BODIPYFL) and nAChRγGFP. First, WT nAChRs were used to determine the αBtxAlexa488 baseline level of labeling and the photobleaching step distribution. The WT nAChR was expressed at similar levels as nAChRb19LyS(BODIPYFL), as confirmed by ACh-induced currents (\(I_{\text{max}} \approx \sim 2\) mA). The WT nAChR labeled with αBtxAlexa488 displayed puncta (Fig. S2A) with a log-normal amplitude distribution with a peak value at 773 counts and a log SD of 0.45 (\(n = 2\) oocytes, 149 puncta; 43 mW/cm\(^2\)), and the peak value increased to 1157 counts with a log SD of 0.43 at a higher power density (\(n = 1\) oocyte; 102 puncta; 69 mW/cm\(^2\)) (Fig. 6A). All photobleaching step analysis was restricted to a relatively low power density of 43 mW/cm\(^2\), to resolve multiple steps before photobleaching. The WT nAChR labeled with αBtxAlexa488 resulted in 1 (48% ± 2%), 2 (43% ± 3%), 3 (6% ± 1%), and 4 (3% ± 1%) detected photobleaching steps (\(n = 2\) oocytes, 144 puncta) (Fig. 6B). Despite the two αBtx sites per receptor, WT receptors labeled with αBtxAlexa488 displayed a plurality (~48%) of single bleach steps under all conditions. We ascribe this result to the incomplete labeling of αBtxAlexa488 (~74% of αBtx are labeled by a fluorescent Alexa488; see Methods) and to the unintentional photobleaching of fluorescent molecules during sample-handling and a brief fine focus before image acquisition. Thus, if (1), ~20% of puncta contain two receptors and 80% contain one receptor, as based on results from nAChRb19LyS(BODIPYFL) and nAChRγGFP, and (2), 74% of the αBtx have a fluorescent Alexa488, then the theoretical binomial fluorescent dye distribution per puncta is: 0 (6%), 1 (32%), 2 (48%), 3 (8%), and 4 (6%). Excluding the nonmeasurable nonfluorescent dyes (0 steps), the theoretical photobleaching step distribution becomes 1 (34%), 2 (51%), 3 (9%), and 4 (6%). However, Alexa488 photobleaches during fine focus. The measured photobleaching step distribution is adequately explained by assuming that the original 74% of fluorescent αBtx molecules decreased to 57%: the theoretical observable distribution becomes 1 (49%), 2 (41%), 3 (8%), and 4 (2%). We use the following assumptions throughout our step photobleaching analysis: 20% of diffraction-limited puncta contain two nAChRs, and 57% of the αBtx contain fluorescent Alexa488.

The nAChRγGFP were labeled with αBtxAlexa488 (Fig. 7A and Fig. S2B), and we detected a distribution of 1 (40% ± 3%), 2 (33% ± 2%), 3 (18% ± 3%), and ≥4 (9% ± 3%) photobleaching steps (\(n = 2\) oocytes, 148 puncta) (Fig. 7B). Interestingly, the puncta density ratio between the nAChRγGFP receptors and the receptors labeled with αBtxAlexa488 was 0.33 (\(n = 4\); two oocyte batches). The apparent underreporting by the γGFP label, compared with αBtxAlexa488, arises from at least two factors. The first is unintended photobleaching during fine focus: eGFP
bleaches more quickly than Alexa488. The second is that 100% of eGFP molecules reach the mature fluorescent state (2). Again, if we assume that a BtxAlexa488 is reporting the presence of only 57% of receptors, then the expected binomial distribution for photobleaching steps is: 1 (36%), 2 (41%), 3 (15%), and 4–6 (8%).

As expected, a subset of puncta containing the nAChR/ Lys(BODIPYFL) labeled with a BtxAlexa488 yielded three discrete photobleaching steps, in agreement with the predicted stoichiometry (Fig. 8, A and B, and Fig. S2 C). The oocytes injected with nAChR β19′GGGU mRNA and YFaFSACCC-Lys(BODIPYFL), and incubated with a BtxAlexa488, displayed puncta with 1 (44% ± 8%), 2 (36% ± 2%), 3 (18% ± 8%), and 4–6 (2% ± 1%) photobleaching steps (n = 3 oocytes, 194 puncta). The puncta density ratio between nAChR β19′Lys(BODIPYFL) and nAChR β19′Lys(BODIPYFL) labeled with a BtxAlexa488 was 0.47. If the probability of a punctum having one detectable Lys(BODIPYFL) per nAChR is 47%, then the binomial distribution for measurable photobleaching steps is: 1 (31%), 2 (41%), 3 (19%), and 4–6 (9%). Thus, the measured photo-

bleaching-step percentages are within ~13% of values expected from straightforward molecular labeling (Fig. 9 A).

**Fluorescent puncta density and current density analysis**

We observed that the optimal single-molecule TIRF microscopy range was $I_{\text{max}} = 1–5 \mu A$ for WT nAChRs (subsequently labeled with a BtxAlexa488) and for nAChR γeGFP. In oocytes with ACh-induced currents <1 μA, no nAChR γeGFP receptors were detected. Thus, we compared puncta density with recorded currents. We made the following assumptions: 1), The nAChRs were distributed homogenously throughout the oocyte plasma membrane. 2), The oocyte plasma membrane uniformly adhered to the glass coverslip during single-molecule TIRF imaging, so that the measured image frames can be extrapolated to the entire oocyte. 3), Stage VI Xenopus oocyte plasma membrane capacitance is 0.25 μF (reported range, 0.19–0.25 μF) (60,61). 4), The specific membrane capacitance is 0.8 μF/cm² (62), implying that
the average total oocyte plasma membrane area is $3.13 \times 10^7 \mu m^2$. 5). Desensitization was negligible during the growth phase of ACh-induced currents, so that $I_{max}$ measures the simultaneous activation of all receptors. 6). Receptors in microvilli that contribute to capacitance measurements are retained during imaging (63). Additional experimental details were as follows: 7). The image frame area was 1758.1 $\mu m^2$. 8). The oocyte holding potential was $-60$ mV. 9), Under the ionic conditions of our experiments, the single-channel nAChR conductance was 36 pS (64).

In experiments on WT nAChRs, $I_{max} = 1.4 \pm 0.5 \mu A$, implying an expected density of 0.027 receptors/$\mu m^2$. When these oocytes were labeled with $\alpha$BtxAlexa488, we measured $0.036 \pm 0.01$ puncta/$\mu m^2$ (range, $0.017$--$0.058/\mu m^2$; $n = 3$ oocytes) (Fig. 9 B).

In experiments with nAChR $\gamma_{eGFP}$, $I_{max} = 1.5 \pm 0.8 \mu A$, leading to an expected receptor density of $0.029/\mu m^2$. The measured puncta density was $0.020 \pm 0.006/\mu m^2$ (range, $0.013$--$0.032/\mu m^2$; $n = 3$ oocytes) (Fig. 9 B). Other oocytes from this nAChR $\gamma_{eGFP}$ batch were also labeled with $\alpha$BtxAlexa488. The measured puncta density for nAChR $\gamma_{eGFP}$ labeled with $\alpha$BtxAlexa488 was $0.059 \pm 0.03/\mu m^2$ (range, $0.018$--$0.126/\mu m^2$; $n = 3$ oocytes) (Fig. 9 B). Puncta with single-molecule profiles were observed at a lower density for relevant control oocytes: the measured puncta density for un.injected and unlabeled oocytes was $0.0003 \pm 0.0003$ puncta/$\mu m^2$ (range, $0.0$--$0.001/\mu m^2$; $n = 4$ oocytes) (Fig. S1 D).

The average puncta density for coinjection of nAChR $\beta1'GGGU$ mRNA (25 ng/oocyte) and 12.5 ng YFAR-SACCC-Lys(BODIPYFL) was $0.021 \pm 0.003/\mu m^2$ (range, $0.015$--$0.028$; $n = 5$ oocytes). The expected puncta density was $0.068$ puncta/$\mu m^2$ for this batch ($I_{max} = 3.5 \pm 0.2 \mu A$, $n = 4$ oocytes) (Fig. 9 B). When these oocytes expressing nAChR $\beta1'LYs$ (BODIPYFL) receptors were labeled with $\alpha$BtxAlexa488 (Fig. S2 C), the puncta density was $0.045 \pm 0.009/\mu m^2$ (range, $0.027$--$0.07/\mu m^2$; $n = 6$ oocytes) (Fig. 9 B). Thus, as expected from the data regarding labeling and dye photobleaching (Figs. 3 A and 8 A), the di-labeling of nAChRs revealed receptors that went undetected in experiments incorporating Lys(BODIPYFL). Control oocytes from the same batch were injected (as usual) with YFAR-SACCC-Lys(BODIPYFL) (12.5 ng) (i.e., with tRNA-fUAA but no mRNA), to assess how unincorporated Lys (BODIPYFL) contributed to the observed puncta. In these control oocytes, puncta were detected at a much lower average density of $0.007 \pm 0.002/\mu m^2$ (range, $0.003$--$0.014/\mu m^2$; $n = 5$ oocytes) (Fig. 9 B and Fig. S1 B). When other similarly injected control oocytes were labeled with $\alpha$BtxAlexa488, we measured a puncta density of $0.004 \pm 0.002/\mu m^2$ (range, $0.002$--$0.008/\mu m^2$; $n = 3$ oocytes) (Fig. 9 B and Fig. S2 D). Another type of control used uninjectected oocytes incubated with $\alpha$BtxAlexa488; the puncta density was $0.003 \pm 0.002/\mu m^2$ (range, $0.0$--$0.006$; $n = 3$ oocytes) (Fig. 9 B and Fig. S2 E). Thus, the experimental values were all internally consistent within a factor of 3.2, which is acceptable, given the number of assumptions made. Two types of control yielded much lower puncta densities (Fig. 9 B).

DISCUSSION

We performed imaging of Xenopus oocytes heterologously expressing nAChR $\beta1'LYs$ (BODIPYFL) in the plasma membrane. Background signals were minimized by using
the frameshift-suppressor tRNA (YFaFSACC) (37,51,52). The frameshift strategy was crucial, because the more common nonsense-suppression strategy, using the amber suppressor tRNA (THG73), produced unacceptably high background signals (data not shown). The β19' site is buried in the resting state of the receptor (Fig. 1 C), and the efficient incorporation of Lys(BODIPYFL) demonstrates an advantage over genetic FP insertions or posttranslational labeling, such as BODIPYFL-C3-MTS (Fig. 1 E) labeling of β19'C, which was not possible. Plasma membrane imaging of channels incorporating fUAAs with single-molecule TIRF microscopy may be considered a technique that extends beyond the incorporation of fUAAs in receptors and subsequent purification and reconstitution.

The puncta originating from nAChR β19’Lys (BODIPYFL) were brighter than those puncta from nAChR γeGFP, by an average factor of ~2 (Fig. 5), in agreement with the known photophysics of BODIPYFL and eGFP in solution (2,15,36). This is surprising, considering that Lys (BODIPYFL) is incorporated in the interior of an ion channel (Fig. 1 C) and interacts with many other amino acids. Also, labeling with the highly specific fluorescent ligand, αBtxAlexa488, allowed us to confirm that nAChR molecules produced most of the puncta from oocytes expressing nAChR β19’Lys(BODIPYFL) and nAChR γeGFP receptors (Figs. 7 and 8). The αBtxAlexa488 studies suggest that ~47% of the predicted Lys(BODIPYFL) are detected. Thus, similar to FPs (65,66) and quantum dots (67), there is a nonfluorescent fraction of molecules, but there are partially different reasons for each fluorophore. Dye photobleaching during synthesis, handling, expression, and focusing of a subset of nAChR β19’Lys(BODIPYFL) are the primary reasons for the dark nAChR fraction. Although our data on αBtxAlexa488 labeling might be limited by impurities in the commercial αBtxAlexa488, the resolved multistep photobleaching was consistent with the known 2:1 αBtxAlexa488/nAChR stoichiometry (59).

In general, the single-molecule labeling schemes based on genetically encoding (fUAA and eGFP) yield a ratio, (puncta density)/(receptor density expected from electrophysiology), between 0.5 and 2. The experiments with the tightly bound ligand, αBtxAlexa488, gave a ratio of 3–4. We consider this agreement quite satisfactory, considering the numerous assumptions 1–6 (above in Fluorescent Puncta Density and Current Density Analysis). The measured ratio would be changed in unknown directions by variable adherence. Although the puncta density differed between mono-labeled and di-labeled receptors because of photobleaching, many viable single fluorophores were still present, enabling parallel single-molecule measurements.

An important advantage of small-molecule fluorophores is that they are less likely than fluorescent proteins to perturb protein structure, function, and trafficking (see comparison of FP size in Fig. 1 A compared with Lys(BODIPYFL) in Fig. 1 B). This study describes a methodology to incorporate small-molecule fluorophores directly into nascent proteins, using UAA incorporation technology, and allows for the detection of single-membrane proteins in live cellular membranes. Site-specific and single-step fUAA incorporation will facilitate investigations of many membrane proteins, but are particularly useful in measuring the gating dynamics, colocalization, and trafficking in ion channels, because these channels provide complementary single-molecule measurements of function. The incorporation of fUAAs is particularly important for sites within α-helices (Fig. 1 D) and within other structural motifs that may not tolerate the large perturbation of fluorescent proteins (Fig. 1 A).

Finally, although the focus of our report is live-cell imaging, recent reports describe the super-resolution microscopy of fixed cells (45,68). The localization of fluorescent molecules with ~2-nm resolution was reported. The resolution of single molecules is dependent on dye brightness (69). In addition, it was proposed that the power of super-resolution microscopy will be significantly enhanced with genetically encoded synthetic dyes into proteins (70). The puncta originating from nAChR β19’Lys(BODIPYFL) were brighter than those puncta from nAChR γeGFP, by an average factor of ~2 (Fig. 5). Thus, Lys(BODIPYFL) is an attractive alternative to FPs for fixed-cell super-resolution microscopy.

SUPPLEMENTARY MATERIAL

Two figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(08)00012-X.

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