Supporting Information

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SI Materials and Methods

Synthesis of N-AlphaboC-4-Bromo-l-Tryptophan Methyl Ester. The 4-bromo-L-tryptophan (283 mg, 1 mmol) was dissolved in 20% MeOH in DCM (10 mL) and trimethyloxylidiazomethane (2 M in hexane) was added dropwise at room temperature. The solution was stirred for 3 h and the volatiles were removed under reduced pressure. The residue was dissolved in anhydrous DCM (2 mL) then triethylamine (2 mmol) and Boc anhydride (0.23 mL, 1.1 mmol) were added. The mixture was stirred overnight and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0–10% MeOH in DCM) to yield N-alphaboC-4-bromo-l-tryptophan methyl ester (192 mg, 48%). The expected mass (MH\(^+\)) of 397.7 was observed using ESI-MS (positive).

Synthesis of N-AlphaboC-4-Cyano-4-Tryptophan Methyl Ester. A mixture of N-alphaboC-4-cyano-4-tryptophan methyl ester (192 mg, 0.48 mmol), Zn(CN)\(_2\) (38 mg, 0.33 mmol), and tBuXPhos Pd G3 (2 mL) and ethyl acetate (38 mg, 0.33 mmol), and tBuXPhos Pd G3 solution (2 mL) and ethyl acetate (2 mL) were added. The mixture was vigorously stirred at 40 °C overnight under N\(_2\). Then, a saturated NaHCO\(_3\) solution (2 mL) and ethyl acetate (3 mL) were added, stirred for 5 min, and separated. The aqueous layer was extracted with ethyl acetate and the combined organic layer was dried over Na\(_2\)SO\(_4\). The volatiles were removed under reduced pressure and the residue was purified by silica gel column chromatography (10–70% ethyl acetate in hexanes) to give the desired product (16 mg, 10%).

The following proton (\(\text{H}\)) NMR peaks at 300 MHz in DMSO-D\(_6\) were observed: δ ppm 11.53 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.49 (d, J = 7.1 Hz, 1H), 7.40 (s, 1H), 7.31 (d, J = 7.4 Hz, 1H), 7.21 (t, J = 7.8 Hz, 1H), 4.30–4.25 (m, 1H), 3.59 (s, 3H), 3.40–3.35 (m, 1H), 3.20–3.14 (m, 1H), 1.31 (s, 9H). The expected mass (MNa\(^+\)) of 366.4 was observed using ESI-MS (positive).

Synthesis of N-AlphaboC-4-Acetyl-4-Cyano-Trp Methyl Ester (4CN-Trp). N-alphaboC-4-cyano-4-tryptophan methyl ester (16 mg, 0.047 mmol) was treated with 50% trifluoroacetic acid in DCM (2 mL) for 1 h. The volatiles were removed under reduced pressure and the sample was lyophilized. The product was purified by reverse phase HPLC. N-terminal acetylation was accomplished by stirring the amino acid in acetic anhydride (5:1 molar ratio) in DMF for 1 h. The sample was then dissolved in H\(_2\)O and lyophilized to remove the DMF and water. Purified amino acid was purified by reverse phase HPLC and identification and verification of the desired product (16 mg, 10%).

The expected mass (MH\(^+\)) of 386 was observed using ESI-MS (positive).

Materials and Sample Preparation. L(−)-Tryptophan (99%, Acros Organics), 4-cyanoindole (4CNI) (97%, Acros Organics), 4-cyanoindole-3-acetic acid (4CNI-3AA) (98%, Ark Pharm), 9,10-diphenylanthracene (DPA) (98%, Acros Organics), and enhanced blue fluorescent protein (EBFP) (97%, BioVision, Inc.) were used as received. 4CN-Trp-Gly and 4CN-Trp-Met were synthesized manually and 4CN-Trp-MpX (Sequence: 4CN-Trp*-INWKGIAMAKKL-NH\(_2\)) was synthesized on a Liberty Blue Automated Microwave Peptide Synthesizer (CEM Corporation); all used standard Fmoc-based solid phase peptide synthesis to couple the 4CN-Trp\(^{\*}\) fluorophore to the N terminus of the peptide of interest via the carboxylic acid on 4CNI-3AA. Purification of peptides was achieved using reverse phase HPLC and identification and verification of the peptides were done using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Stock solutions of Trp, 4CNI, and 4CNI-3AA were made by dissolving the respective compound in Millipore water and the solute concentration was determined by weight. The DPA samples in cyclohexane were prepared similarly. The concentrations of 4CN-Trp\(^{\*}\)-containing peptides and 4CN-Trp samples in Millipore water were determined optically at 305 nm using the extinction coefficient determined for 4CNI-3AA (5780 M\(^{-1}\) cm\(^{-1}\) in water) (Fig. S4).

Cell Culture. HEK293T/17 cells were seeded in an eight-well Lab-Tek chamber slide at 3,000 cells per well. Cells were grown overnight at 37 °C in a 5% CO\(_2\) incubator. Before use, the growth media was removed by pipette from the cells and the cells were subsequently washed once with pH 7.4 phosphate buffer saline (PBS) (Thermo Fisher). An appropriate amount of a 500 \(\mu\)M stock solution of 4CN-Trp\(^{\*}\)-MpX in Millipore water was added to each well containing the cells in PBS to achieve the desired final peptide concentration (1 or 10 \(\mu\)M). The mixture was allowed to incubate for 1 min before wide-field imaging measurements. For cell samples used in the confocal microscopic measurement, the mixture was incubated ~10 h at room temperature before use.

Absorption Measurements. UV-Vis absorption spectra were collected on a Jasco V-650 UV-Vis spectrophotometer using a 1.0-cm quartz cuvette at room temperature.

Static and Time-Resolved Fluorescence Measurements. All static fluorescence measurements were collected on a Jobin Yvon Horiba Fluorolog 3.10 fluorometer at 25 °C (or 37 °C, as noted) using a 1.0-cm quartz cuvette with spectral resolution of 1.0 nm and an integration time of 1.0 s/nm. To minimize the inner filter effect, self-quenching, and potential detector saturation, each and every solution used in the QY measurements was prepared by diluting a stock solution of high concentration (the absorbance at the corresponding excitation wavelength was in the range of 0.1–0.5) 100-fold. QYs were determined as described in the text and for each case, the reported value corresponds to the average of three measurements using independent solutions. Additionally, for the QY determination of 4CNI-3AA, 4CN-Trp*, and Gly-Gly-4CN-Trp at 325 nm, the gradient method was used where a series of dilutions was used to determine the ratio between the integrated area of the fluorescence, I, and the absorbance at the \(\lambda_{ex}\), A (Fig. S5). This ratio is then used in Eq. 1 in the text to calculate the QY.

For the photobleaching experiments, EBFP and 4CN-Trp\(^{\*}\)-Gly solutions were prepared in water such that the integrated optical densities for both samples are the same (~0.5) in the spectral
region of the excitation light (355 ± 2.5 nm), defined by the excitation slit width of the Fluorolog 3.10 fluorometer that uses a 450-W Xe arc lamp as the light source. Emission intensity was recorded at 450 ± 1 nm for EBFP and at 415 ± 1 nm for 4CN-Trp*-Gly every minute for 10 h.

Time-resolved fluorescence measurements were obtained on a time-correlated single photon counting (TCSPC) system with a 0.4-cm quartz cuvette at room temperature. The details of the TCSPC system have been described elsewhere (16). Briefly, the 270-nm excitation pulse was generated by collinear frequency tripling of the fundamental output (800 nm) of a home-built femtosecond Ti: Sapphire oscillator (85 MHz). The repetition rate was decreased to 21 MHz using an electro-optical pulse picking system (Conoptics, Inc.). Fluorescence decays were collected under the magic-angle polarization condition using a microchannel plate photomultiplier tube (MCP-PMT) detector (Hamamatsu R2809U) and a TCSPC board (Becker and Hickl SPC-730). Rejection of excitation light was accomplished by a 405/50-nm bandpass filter (Semrock) and a 300-nm longpass filter (Semrock). Fluorescence decays were deconvoluted with the experimental instrument response function (IRF) and were fit to either a single-exponential or biexponential function using FLUORFIT (Picoquant GmbH). The OD of each sample was ∼0.1 at the excitation wavelength (270 nm).

**Fluorescence Imaging.** Wide-field fluorescence images were acquired at room temperature using an Olympus IX71 inverted microscope equipped with a 100-W Hg lamp, a 60× (0.9 N.A.) water objective, and HCImage Live software. Excitation light was selected using a 355/25-nm bandpass filter and the emission was isolated via a 420-nm longpass filter. The integration time for each image was 50 ms. Image processing and analysis were carried out using the ImageJ 1.50 software (34). Confocal images were collected on a home-built stage-scanning confocal microscope at room temperature. Specifically, an inverted microscope (Eclipse TE300, Nikon) equipped with a 100× oil immersion objective (CFI S Fluor, Nikon) was used to condense the excitation light, which was derived from a UV LED light source (M340L4, Thorlabs). This LED produces continuous wave (CW) light centered at 344 nm with a full width at half maximum (FWHM) bandwidth of 10 nm. The light emitted from the LED was first collimated by a collimation adapter (SM1P25-A, Thorlabs), followed by further collimation of the resultant beam using a two-lens collimator with a 60-μm pinhole at the cofocal point of the two focusing lenses. The power of the incident light was measured to be ∼6 μW before entering the microscope. A dichroic mirror with an edge wavelength of 349 nm (ZT349rdc, Chroma Technology) and a longpass filter with a cutoff wavelength of 365 nm (ET365lp, Chroma Technology) were used to allow the pass/rejection of the emission/excitation light. Fluorescence emission was then focused and passed through a 60-μm confocal pinhole and then measured by a single photon counting detector (SPCM-AQRH-15, Excelitas Technologies) with an integration time of either 0.5 or 1.0 ms at each scanning position. The confocal image was acquired by a point scanning method, which was accomplished by a high-resolution XY microscope stage (P-734, Physik Instrumente) controlled by a piezo controller (E-501.00, Physik Instrumente).

**Fig. S1.** (A) Fluorescence spectra (λ<sub>ex</sub> = 325 nm) and (B) fluorescence decay kinetics (λ<sub>ex</sub> = 270 nm) of 4CN-Trp*-Gly and 4CN-Trp*-Met, as indicated. The optical densities of both samples at λ<sub>ex</sub> were equal. Fitting each fluorescence decay in B to a single-exponential function (black) yielded a lifetime of 12.6 ns for 4CN-Trp*-Gly, and 12.2 ns for 4CN-Trp*-Met. The residuals to the fits are shown at Top.
Fig. S2. Fluorescence decay kinetics of (A) 4CN, (B) 4CN-3AA, (C) 4CN-Trp*-Gly, (D) 4CN-Trp*-Gly in THF, (E) 4CN-Trp, and (F) Gly-Gly-4CN-Trp, all in H2O except D. In each case, the smooth black line corresponds to the best fit of the respective data to a single-exponential (A–D) or a biexponential (E and F) function and the resultant lifetime(s) is given in Table 1. Top in each case is the corresponding residual plot.

Fig. S3. Fluorescence microscopic images of HEK293T/17 cells in the presence of 10 μM 4CN-Trp*-MpX obtained at different time points, as indicated. Between each measurement, the sample was continuously illuminated by the excitation light of the microscope. It is noticeable that the fluorescence intensity undergoes a large change between the first and second image. However, it is unclear whether this change is caused by photobleaching or peptide redistribution between the solution and cellular phases.
Fig. S4. Beer’s law plots of (A) 4CNI and (B) 4CNI-3AA at the indicated wavelengths. The straight line in each case corresponds to a linear regression of the respective data with a zero y-intercept, which yielded a molar extinction coefficient of \(8,056 \pm 24 \text{ M}^{-1} \cdot \text{cm}^{-1}\) for 4CNI at 305 nm, and \(5,778 \pm 8 \text{ M}^{-1} \cdot \text{cm}^{-1}\) and \(4,750 \pm 8 \text{ M}^{-1} \cdot \text{cm}^{-1}\) for 4CNI-3AA at 305 nm and 330 nm, respectively.

Fig. S5. Integrated fluorescence intensity of DPA, 4CNI-3AA, 4CN-Trp, and Gly-Gly-4CN-Trp as a function of absorbance at 325 nm. The solid color line in each case corresponds to a linear regression of the respective data with a zero y-intercept, which yielded a slope of \((1.80 \pm 0.07) \times 10^{10}\) for DPA, \((1.89 \pm 0.04) \times 10^{10}\) for 4CNI-3AA, \((2.02 \pm 0.04) \times 10^{10}\) for 4CN-Trp, and \((2.00 \pm 0.03) \times 10^{10}\) for Gly-Gly-4CN-Trp. The calculated QYs at 325 nm are shown in Table 1.