

Supplementary Material For:

Detection of SNARE complexes with FRET using the tetracysteine system

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Fluorimetry

Fluorescent studies were performed using the PTI spectrofluorimeter (Photon Technology International, Canada) and Felix-32 software. Cerulean fluorescence was excited at 433 nm and emission spectra were collected in the range of 450-600 nm with the integration time 0.1 s. FAsH fluorescence was excited at 500 nm and emission spectra were collected in the range of 510-600 nm with the integration time 0.1 s. Excitation and emission slits were set to 5 nm. Typically, 40 μ M stock solution of CSNAC was thawed on ice, and 5 μ l of it was transferred in the 10 mm quartz cuvette (Starna Cells, Inc) supplied with a stir bar and 1 ml of 20 mM HEPES, 0.1 M KCl pH 7.4 to the final protein concentration of 0.2 μ M. Under these experimental conditions, background signal due to the Raman-scattering was negligible. At CSNAC concentrations less than 10 nM, the 504 nm Raman peak may interfere with low fluorescence signals.

The binding efficiency of FAsH to CSNAC

Binding efficiency of FAsH was determined by labeling 40 μ M solution of CSNAC2 with 0 μ M, 2.5 μ M, 5 μ M, 10 μ M, 20 μ M, and 40 μ M FAsH. CSNAC was labeled with indicated concentrations of FAsH, as described above, and binding stoichiometry was determined using two independent methods. The first approach assumes that the number of emitted photons (Em) depends on the number of absorbed photons (Abs) and the quantum yield of chromophore (Φ):

$$Em = \Phi \times Abs \quad [\text{Eq. 1}]$$

According to the Beer-Lambert law:

$$Abs = \epsilon cl \quad [\text{Eq. 2}]$$

Where ϵ is the molar absorption coefficient, c is concentration, and l is the pathlength. By solving equations 1 and 2,

fluorophore concentrations can be determined from Em values:

$$c = Em / \Phi \epsilon \quad [\text{Eq. 3}]$$

Given that $l = 1$ cm, $\Phi_{\text{Cerulean}} = 0.62$, $\Phi_{\text{FAsH}} = 0.85$, $\Phi_{\text{Cerulean}} = 43,000 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{\text{FAsH}} = 70,000 \text{ M}^{-1} \text{ cm}^{-1}$, the calculated FAsH:CSNAC molar ratio varied between 0.7:1 and 0.8:1 (an example shown in Figure 1F). The limitation of this method is that ϵ_{FAsH} may depend on the local amino acid environment imposed by the tetracysteine peptide. The second method determines FAsH content in labeled CSNAC preparations using the binding curve. For each preparation of labeled proteins, emission spectra of Cerulean and FAsH were collected by direct excitation at 433 nm and 500 nm, respectively, and emission ratios Em530/Em475 were plotted against FAsH concentrations. Maximal binding (Em530/Em475) was determined using a single exponential fit (Wolfram Mathematica-8). This method indicates that FAsH is bound to CSNAC2 at 95% of the theoretical maximum (an example shown in Figure 1F).

FRET calculations

FRET efficiency (E) was determined as:

$$E = 1 - [I_{\text{DA}} / I_{\text{D}}] \quad [\text{Eq. 4}]$$

Where I_{DA} is emission intensity of the donor in the presence of FAsH and I_{D} is emission intensity of the donor in the absence of FAsH (FAsH can be removed from the tetracysteine peptide by addition of 5 mM 2,3-Dimercapto-1-propanol (BAL) to a fluorimeter cuvette for 5 minutes). This approach allows real-time measurements of E values using the same incubation mixture. Forster radius (R_0) was calculated as:

$$R_0 = (8.79 \times 10^{-5} \times n^4 \times \Phi_{\text{Cerulean}} \times J(\lambda) \times k^2)^{1/6} \quad [\text{Eq. 5}]$$

Where n is the refractive index, Φ_{Cerulean} is quantum yield of a donor, $J(\lambda)$ is the overlap integral between the Cerulean emission spectrum and FAsH excitation spectrum, and k^2 is the orientation factor. The distance between fluorophores (R) was calculated from:

$$E = [1 + (R_0/R)^6]^{-1} \quad [\text{Eq. 6}]$$