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Video Article

Visualizing Intracellular SNARE Trafficking by Fluorescence Lifetime Imaging Microscopy

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Abstract

Soluble *N*-ethylmaleimide sensitive fusion protein (NSF) attachment protein receptor (SNARE) proteins are key for membrane trafficking, as they catalyze membrane fusion within eukaryotic cells. The SNARE protein family consists of about 36 different members. Specific intracellular transport routes are catalyzed by specific sets of 3 or 4 SNARE proteins that thereby contribute to the specificity and fidelity of membrane trafficking. However, studying the precise function of SNARE proteins is technically challenging, because SNAREs are highly abundant and functionally redundant, with most SNAREs having multiple and overlapping functions. In this protocol, a new method for the visualization of SNARE complex formation in live cells is described. This method is based on expressing SNARE proteins C-terminally fused to fluorescent proteins and measuring their interaction by Förster resonance energy transfer (FRET) employing fluorescence lifetime imaging microscopy (FLIM). By fitting the fluorescence lifetime histograms with a multicomponent decay model, FRET-FLIM allows (semi-)quantitative estimation of the fraction of the SNARE complex formation at different vesicles. This protocol has been successfully applied to visualize SNARE complex formation at the plasma membrane and at endosomal compartments in mammalian cell lines and primary immune cells, and can be readily extended to study SNARE functions at other organelles in animal, plant, and fungal cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56745/

Introduction

Membrane trafficking is a central feature of eukaryotic cells, where membrane vesicles bud off from a donor organelle and then move to and fuse with a target organelle[1,2]. Except for mitochondria, all these membrane fusion steps are catalyzed by members of the SNARE protein family[1,2]. The SNARE protein family consists of about 36 members in mammalian cells and about 20 members in yeast[2]. SNARE proteins contain one or two ~52 residues-long, natively unstructured regions, called SNARE-motifs. SNARE proteins are often tethered to membranes by a C-terminal transmembrane helix[1,2]. SNAREs can be categorized based on the central residues they contribute to the SNARE complex into arginine (R) and glutamine (Q) SNAREs[1,2]. Membrane fusion is driven by the interaction of 3 or 4 cognate SNAREs that together contribute 4 SNARE-motifs and are distributed over both the donor and acceptor membrane[1,2]. A SNARE complex consists of one R-SNARE motif and three Q-SNARE motifs (termed Qa, Qb, and Qc). Complex formation starts at the N-termini of the SNARE-motifs, forming a so-called *trans*-SNARE-complex, and proceeds towards the C-termini, forming a tight α-helical coiled-coil bundle called a *cis*-SNARE-complex. The complex formation of even a single SNARE complex drags the donor and acceptor membranes together and overcomes the energy barrier for membrane fusion[3].

Assigning distinct SNARE complexes to specific transport routes within cells is often technically challenging. Although SNAREs clearly contribute to the specificity of membrane trafficking, they are promiscuous, functionally redundant, and their functions overlap[1,2]. Because of this, perturbation experiments targeting SNAREs, such as by gene knockout, RNA interference, the introduction of blocking antibodies, or with soluble SNARE fragments acting as dominant negatives, frequently do not result in clear phenotypes as other SNAREs compensate[2,4]. Moreover, it is difficult to differentiate specific membrane fusion steps from upstream trafficking events, because SNAREs can be involved in multiple transport routes[2]. Localization studies of SNAREs by microscopy approaches, using immunolabeling or genetic fusion to fluorescent reporter proteins, suffer from the problems that: (i) SNAREs locate to multiple organelles as they often mediate multiple trafficking steps, and (ii) their localizations do not automatically mean they are functionally engaged in SNARE complex formation. Finally, SNARE complexes can be identified using immunoprecipitation experiments using one of the SNAREs as bait and the other SNAREs as targets, but this does not allow assignment of these complexes to specific organelles or trafficking routes. Thus, currently, there is no alternative technique to visualize SNARE complexes with organellar resolution. Immunofluorescence is not able to prove SNARE interactions but can show only the presence or absence of co-localization, while immunoprecipitation only can show SNARE interactions in the whole cell population but not assign the organelles where these interactions occur.

To overcome these limitation, a novel method allowing for the quantitative visualization of SNARE complexes within live cells with organellar resolution was recently developed by Verboogen *et al.*[5] This method is based on the expression of pairs of SNAREs with spectrally shifted

fluorescent proteins fused C-terminally to their transmembrane helices. After completion of membrane fusion and formation of a *cis*-SNARE complex, these fluorophores at the C-termini of the transmembrane helices are immediately juxtaposed to each other. The fluorophores are then well within the Förster distance (typically <5 nm), resulting in FRET from the green-shifted donor fluorophore to the red-shifted acceptor fluorophore[5,6]. FRET results in quenching of the donor fluorophore and an increased emission of the acceptor fluorophore that can be measured from the ratios of the donor and acceptor emission (ratiometric FRET). However, ratiometric FRET between two different molecules is challenging, because of fluorescence cross-talk and different levels of donor and acceptor SNAREs at different organelles and among cells[7,8]. FRET can also be measured from the fluorescence lifetime, which is the time between the excitation and the emission of a photon. If the donor fluorophore can release its energy by FRET, this competing process result in an apparent shortening of the fluorescence lifetime. This can be measured by FLIM[7,8]. Lifetime FRET is much more robust than ratiometric FRET for measuring interactions between two different molecules, as the fluorescence lifetime is an intrinsic property of a fluorophore and is insensitive to its concentration. Moreover, FRET is by approximation quantitative, because the efficiency of FRET is inversely proportional to the sixth power of the distance between the donor and acceptor fluorophores (essentially a step function). Therefore, by fitting the fluorescence lifetime histograms recorded by FLIM with a double-component decay model, FRET-FLIM allows for the (semi-)quantitative estimation of the fraction of SNARE molecules engaged in SNARE complex formation[5].

Recently, this FRET-FLIM method was used by Verboogen *et al.* to visualize SNARE complex formation in primary dendritic cells of the immune system[5]. It was shown that upon encountering a pathogenic stimulus, dendritic cells reroute their membrane trafficking accompanied by an increased complexing of the R-SNARE vesicle-associated membrane protein (VAMP) 3 with the Qa-SNARE syntaxin 4 specifically at the plasma membrane. This increased SNARE complex formation is likely required to meet the increased secretory capacity for the secretion of inflammatory cytokines such as interleukin-6[5]. This protocol describes the experimental steps needed for the acquisition of FRET-FLIM data for the visualization and (semi-)quantitative measurement of SNARE complexes. It is explained how to fit the whole-cell fluorescence lifetime histograms with mono- and bi-exponential decay functions, resulting in the apparent fluorescence lifetime as a quantitative estimate to SNARE interactions. In this protocol, the widely used HeLa cell line is used as an example, but the method can be readily extended to study SNARE complexes in other eukaryotic cells.

Protocol

1. Preparation of the Microscope Samples

1. Expression of Qa-SNARE syntaxin 4 fused to mCitrine (syntaxin 4-mCitrine; donor fluorophore) and the R-SNARE VAMP3 fused to mCherry (VAMP3-mCherry)
   NOTE: Other SNAREs with fluorescent proteins fused to their C-terminal transmembrane helices can also be used. Instead of mCitrine-mCherry, other donor-acceptor pairs of spectrally separated fluorophores can also be used (*e.g.*, CFP-YFP).
   1. Grow HeLa cells and divide 900,000 HeLa cells over three 35 mm-diameter glass bottom dishes suitable for microscopy.
      NOTE: In principle, this protocol can be adapted for other eukaryotic cell types and microscopy dishes.
      1. Maintain HeLa cell cultures in T75 flasks with 10 mL of high glucose Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic (containing amphotericin B, penicillin, and streptomycin) at 37 °C and 5% CO_2 in a cell culture incubator.
      2. Replenish the medium twice a week and split the cells 1:10 once a week or when the cells reach 85-90% confluency to new T75 flasks (500,000 cells/flask, on average).
      3. Remove the DMEM and wash the HeLa monolayers twice with 8 mL of sterile phosphate-buffered saline (PBS) for 3 min.
      4. Remove the PBS and add 2 mL of PBS with 2 mM ethylenediaminetetraacetic acid (EDTA).
      5. Incubate the cells for 5 min at 37 °C and 5% CO_2 in cell culture incubator and subsequently lightly agitate the flask to separate HeLa cells from the bottom of the flask.
      6. Flush the cells out with 10 mL of medium, transfer the suspension to a 15 mL tube and remove a 10 µL aliquot for counting.
      7. Dilute the aliquot 1:1 with 0.4% trypan blue stain and count the cells with a Bürker hemocytometer[9].
         NOTE: Count two 4 x 4 squares and multiple the cell number by 10,000 for the number of cells/mL.
      8. After cell counting, take 900,000 cells from the 15 mL tube, divide them over the 3 glass bottom dishes (see step 1.1.1), and prepare for transfection.
   2. Transfect the cells by electroporation[10] (see **Table of Materials**) with syntaxin 4-mCitrine construct (donor only, Sample #1), syntaxin 4-mCitrine together with VAMP3-mCherry (Sample #2), and syntaxin 3 fused to both mCitrine and mCherry (Sample #3).
      NOTE: Other cell transfection methods can also be used[11]. The tandem construct in sample #3 is a positive control for estimating the shortest lifetime expectable (*i.e.*, maximal expectable FRET).

2. Culture the cells overnight in high glucose DMEM supplied with 10% FCS and 1% antibiotic-antimycotic at 37 °C with 5% CO_2 in a cell culture incubator.
3. Aspirate DMEM and wash the cells once with live cell imaging medium (140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH = 7.4, mOsm = 300; see **Table of Materials**) for 2 min and subsequently place the cells in fresh imaging medium.

2. Recording of the FLIM Data

1. Place Sample #2 under a time-domain FLIM confocal microscope with a pulsed excitation source for the donor fluorophore (*i.e.*, mCitrine) and time-resolved data acquisition. Keep the cells at 37 °C with a heated stage.
   NOTE: The confocal microscope used in this protocol is equipped with a 63X 1.20 NA water immersion objective, a pulsed white light laser (80 MHz pulsing, < 100 ps pulse duration), a photon multiplier tube (PMT), and a Time-Correlated Single Photon Counting (TCSPC) system (see **Table of Materials** for specific setup used). Other FLIM microscopes can also be used (*e.g.*, single-photon avalanche diodes (SPAD) instead of PMTs, single-wavelength pulsed lasers instead of a white-light pulsed lasers).

2. Select a cell with visible expression of the mCitrine and mCherry-labeled SNARE proteins and record a confocal image of 256 × 256 pixels with simultaneous excitation of both fluorophores prior to each FLIM measurement. A pixel step of about 2-fold smaller than the diffraction limited spatial resolution of the microscope (~ 200 nm) should be used. For mCitrine, excite at 516 nm and collect emission from 521-565 nm; for mCherry, excite at 610 nm and collect emission from 613-668 nm.
   NOTE: Do not position the imaging plane too close to the bottom of the dish (within ~ 2 µm distance), as this will result in a reflection peak in the fluorescence lifetime histogram. Simultaneous excitation of the donor and acceptor fluorophores is preferred, as this allows to overcome the potential problem of sample movement. However, sequential excitation can be used as well.
3. Record a FLIM image by clicking **Run FLIM** with the FLIM image being recorded with the same dimensions and spatial resolution as the confocal image recorded at step 2.2. Record the image at least 50,000 photons for whole-cell FLIM analysis, or at least 400 photons/pixel. Only excite the mCitrine donor fluorophore and not the mCherry acceptor fluorophore, and thus excite at 516 nm and collect emission from 521-565 nm.
4. Repeat steps 2.1-2.3 for multiple cells and for the other Sample #1 (donor only) and Sample #3 (the mCitrine-mCherry tandem construct).
5. Record an instrument response function (IRF). Tune the monochromator of the emission detector to the excitation wavelength (*e.g.*, record emission from 510-550 nm) and then record a FLIM image with the back scattering from a clean glass cover slip by clicking the button **Run FLIM**. Use the same excitation wavelength (516 nm) and laser power as used for recording the cell data at steps 2.1-2.4.
   NOTE: If multiple peaks are visible in the IRF or if the emission monochromators cannot be tuned, the IRF can also be measured with a solution of fluorescent dye with ultrafast lifetime, for instance by fluorescence quenching of an organic dye in a saturated aqueous solution of potassium iodide. Moreover, it is not strictly required to record an IRF, because the decay slope of the fluorescence histograms can also be fitted without deconvolution of the IRF. However, an IRF enables to correct for the timing characteristics of the photon detector used and thereby makes the fits of the lifetime histograms more accurate.

3. Conversion of the Photon Recordings to FLIM Images

1. Download and install the PT32ICS conversion software[5].
   NOTE: The lifetime data can also be analyzed by other software, including software from several microscope vendors.
2. Configure the PT32ICS software.
   1. Set the **Size** to 256 × 256 pixels image size via **Settings | Size**.
   2. Set the **Channel** to **2** via **Settings | Size**.
      NOTE: The channel of the mCitrine emission is exclusively dependent on the configuration of the microscope. A wrong channel will result in a FLIM image with no photons (*i.e.*, black image) and as a result an empty 'LifetimeTable.txt'-file. The correct channel can be identified by trial-and-error.
   3. Set **Output** to **ImageJ (xyz)** (or **TRI2 (xyt)**) for generating FLIM images (step 3.4).
3. Press **Convert** and load one or more photon traces (the .pt3 files). To select multiple photon traces, keep the **Ctrl** key pressed.
   NOTE: The newer 64 bit .ptu format can also be converted.
4. Ensure that the PT32ICS software generates the following files in the same folder as where the pt3 files are saved: one photon stack per .pt3 photon trace in image cytometry standard format (.ics), one bitmap file per .pt3 photon trace (.bmp), a text file per conversion (_Report.txt) containing information on the photon statistics (*i.e.*, the total number of photons in each .pt3 photon trace, the time resolution of detector), and a second text file per conversion (_LifetimeTable.txt) containing the total fluorescence lifetime histograms in tab-delimited format for each .pt3 photon trace.
   NOTE: The .ics file can be used for single pixel fitting in order to generate FLIM images, for instance with TRI2 software[12,13] or the SLIM curve fitting library in FIJI ImageJ[14,15]. This file can also be used for Phasor analysis[16], for instance with the Time Gated Phasor plugin for FIJI from Spechron. The bitmap file contains no lifetime information. The second text file is used for the whole-cell FLIM analysis in step 4.

4. Fitting of Fluorescence Lifetime Histograms for Whole-cell FLIM Analysis

NOTE: This step (deconvoluted fitting of the IRF) requires software capable of deconvoluted fitting. Fitting the slopes of the fluorescence histograms without deconvolution of the IRF can be done with other software as well.

1. Open the data analysis software program capable of fitting with deconvolution (**Table of Materials**) and import the text file containing the histogram for each photon trace (_LifetimeTable.txt) via **File | Import | Single ASCII**.
2. Reorganize the table such that the IRF is in the second column of the table (using **Copy** and **Paste**). Place the IRF in the second column B next to the time values in the first column A.
3. Determine the quality of the recorded photon traces by selecting all columns by pressing **Ctrl + A** and selecting **Plot | Multi-panel | 9 panel**. Do not analyze photon traces with a high reflection peak (**Figure 4F**).
4. Select all columns containing the quality-controlled lifetime histograms that will be fitted as well as the IRF in column B using **Ctrl** key and load the non-linear fitting via **Analysis | Fitting | Nonlinear Curve Fit | Open Dialog**.
5. Load the deconvoluted fit function (available in the **Supplementary File 1**) by adding this function to the analysis software (via **Category | User Defined | Function | Add**). Select the fit function file 'FLIM_convoluted_IRF.fdf'. This function fits the fluorescence lifetime histograms with a mono-exponential decay function deconvoluted with the IRF (*i.e.*, the second column B in the table) (**Equation 1**):

$$y(t) = y_0 + \int A \exp\left(-\frac{t}{\tau}\right) * \text{IRF}(t)\, dt \qquad \text{(Equation 1)}$$

with t the time, τ the apparent fluorescence lifetime, A the amplitude, and y_0 the offset.
NOTE: Another option is to fit the lifetime histograms with a bi-exponential fit function (**Equation 2**):

$$y(t) = y_0 + \int \left(A_1 \exp\left(-\frac{t}{\tau_1}\right) + \int A_2 \exp\left(-\frac{t}{\tau_2}\right)\right) * \text{IRF}(t)\, dt \qquad \text{(Equation 2)}$$

By fixing the lifetime of the slow component (τ_1) to the donor only condition (step 1.1.2: Sample #1) and that of the fast component (τ_2) to the tandem construct (Sample #3), this allows estimation of the fraction of SNARE proteins in complex (F) from the amplitudes of the slow (A_1) and fast components (A_2; see **Discussion**; **Equation 3**):

$$F = \frac{A_2}{A_1 + A_2} \qquad \textbf{(Equation 3)}$$

The fit function file for deconvoluted bi-exponential fitting 'FLIM_convoluted_IRF_biexp.fdf' is available in the **Supplementary File 2**.

6. Select **Fitted Curves | X Data Type** as **Same as Input Data**.
   NOTE: This will result in the appropriate x-axis scaling of the fitted curves.
7. Fit the curves by pressing **Fit**.
   NOTE: This will convert the fit and generate a 'report sheet' in the array with a table containing the fluorescence lifetimes, offsets, and amplitudes. It will also generate a data sheet with the fitted curves and the residuals of the fit.

Representative Results

The rationale of the assay for measuring SNARE interactions by FRET-FLIM is shown in **Figure 1**. The C-termini of the transmembrane helices of cognate SNARE proteins are fused to a pair of spectrally shifted fluorescent proteins (*e.g.*, mCitrine and mCherry). The formation of a *cis*-SNARE complex upon membrane fusion results in these fluorescent proteins becoming immediately juxtaposed to each other and FRET.
Figure 2 shows representative confocal images of HeLa cells expressing fluorescently-labeled SNARE proteins. Shown are a cell expressing syntaxin 4-mCitrine (donor fluorophore) with VAMP3-mCherry (acceptor fluorophore), as well as control conditions of cells expressing only the syntaxin 4-mCitrine construct (donor only; no FRET) and syntaxin 3 fused to both mCitrine and mCherry in tandem (maximal expectable FRET).
Figure 3 shows the accompanying fluorescence lifetime and FLIM images generated by fitting the lifetime histograms of each pixel with mono-exponential decay functions (**Figure 3A-B**) and bi-exponential decay functions (**Figure 3C-D**). **Figure 4A** shows the IRF of our setup measured by back scattering of a microscope cover glass. In **Figure 4B-E**, representative fluorescence lifetime histograms of the whole cell FLIM analysis together with accompanying fit curves for mono-exponential decay functions are shown. **Figure 4F** shows a fluorescent lifetime histogram of an experiment imaged too close to the surface of the microscope cover glass, resulting in a prominent reflection peak. **Figure 4G** shows a lifetime histogram with representative fit with a bi-exponential decay function.
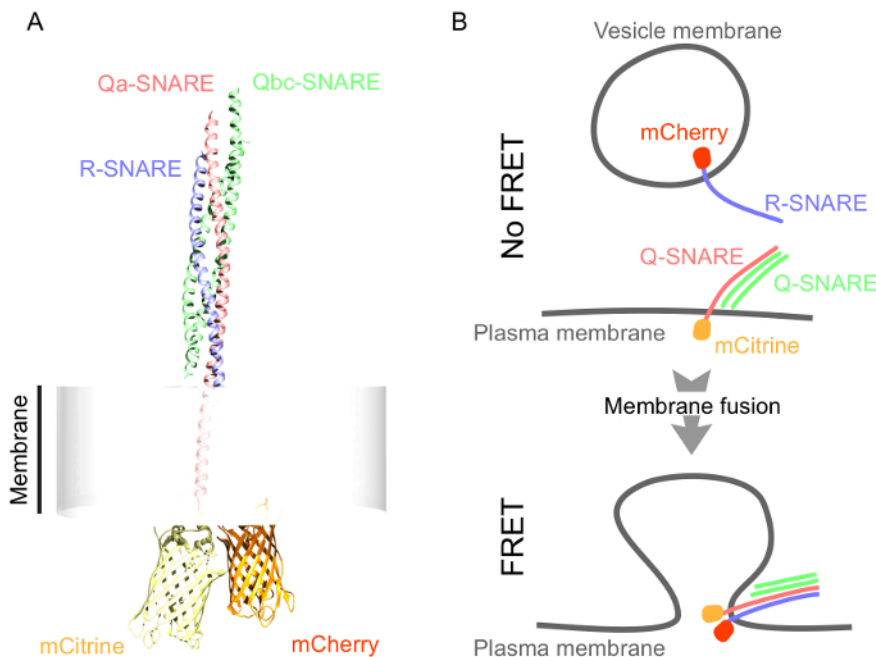

Figure 1: Scheme of the rationale for visualizing SNARE complexes by FRET. (A) Structural model of the neuronal SNAREs (protein database 3HD7[17]) vesicle-associated membrane protein (VAMP) 2 (blue; R), syntaxin 1 (red; Qa-SNARE), and SNAP25 (green; contains both a Qb- and Qc-SNARE motif). The C-terminus of the transmembrane helix of syntaxin-1 is conjugated to mCitrine (donor fluorophore; protein database 3DQ1[18]). The C-terminus of the transmembrane helix of VAMP2 is conjugated to mCherry (acceptor fluorophore; protein database 2H5Q[19]). **(B)** Scheme of SNARE mediated membrane fusion resulting in FRET. After membrane fusion by formation of a *cis*-SNARE complex, the SNAREs are immediately juxtaposed to each other resulting in FRET. Please click here to view a larger version of this figure.
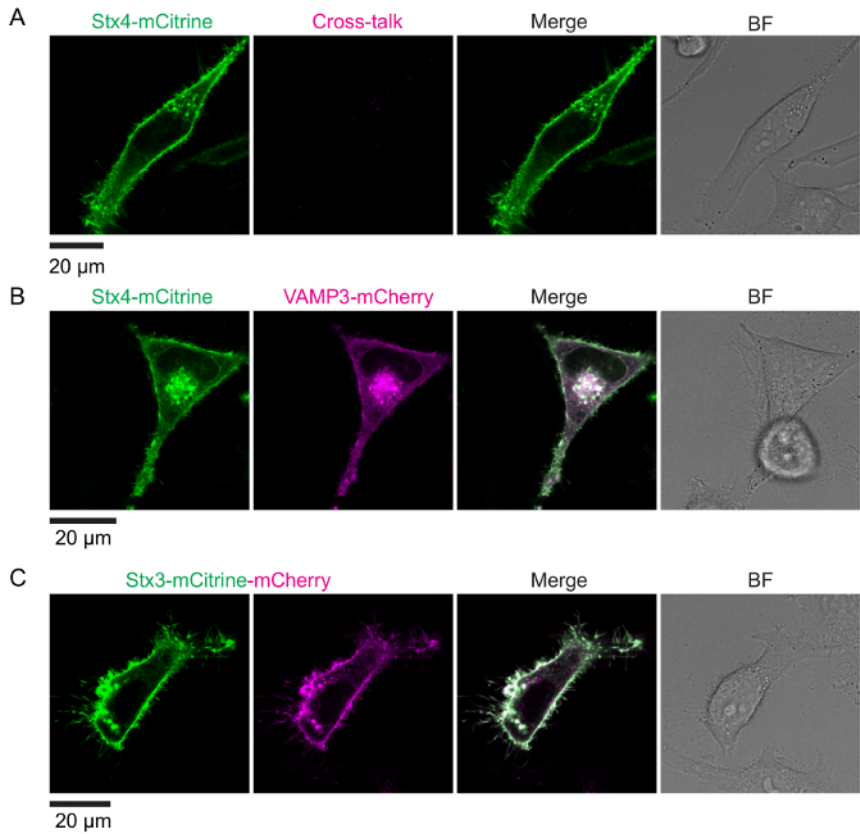
Figure 2: Expression of SNARE proteins fused to fluorescent proteins. First column: donor fluorophore, excited at 516 nm. Second column: acceptor fluorophore, excited at 610 nm. (**A**) Donor only condition. Representative confocal image of a HeLa cell expressing syntaxin 4-mCitrine (donor fluorophore; green in merge). The acceptor channel (second column) shows the fluorescence cross-talk. (**B**) Negative control (no FRET). Representative confocal image of a HeLa cell expressing both syntaxin 4-mCitrine with VAMP3-mCherry (acceptor fluorophore; magenta in merge). (**C**) Positive control (maximal expectable FRET). Representative confocal image of a HeLa cell expressing the syntaxin 3-mCitrine-mCherry tandem construct. Note that the fluorescence signal of the mCitrine and mCherry signals does not completely overlap, likely because of a lower resistance of mCitrine to lysosomal degradation compared to mCherry (see Discussion section). BF: bright field. Scale bars, 20 µm. Please click here to view a larger version of this figure.
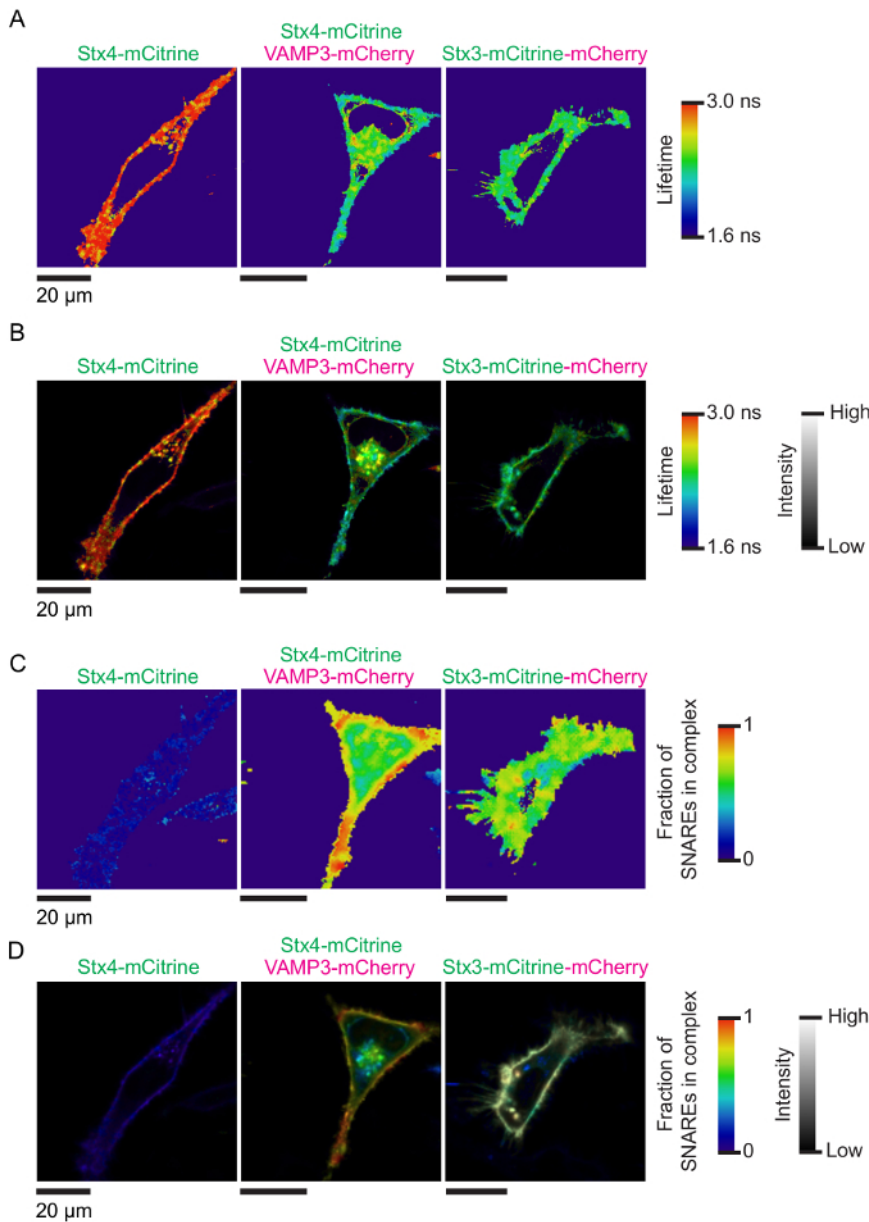
Figure 3: FLIM images of SNARE complex formation. (**A**) Representative fluorescence lifetime images of the cells shown in **Figure 2**. The images were generated by first converting the photon traces recorded by a TCSPC system (.pt3) to the image cytometry standard (.ics) using the PT32ICS software. Single pixel fitted fluorescence lifetime images were then generated using the TRI2 software[12,13] with thresholding from 15-100% intensity, 7 pixel circular binning and monoexponential fitting algorithm (Marquardt). The color indicates the average apparent fluorescent lifetime. (**B**) FLIM images where the fluorescence lifetime images (shown in panel A) were convoluted with the fluorescence intensities of the mCitrine donor fluorophore (shown in **Figure 2**). The convolution was performed with FIJI ImageJ using a custom-made macro (see **Table of Materials**). (**C**) Fluorescence lifetime and FLIM images of the HeLa cell expressing both syntaxin 4-mCitrine and VAMP3-mCherry from panels A-B, but now with fitting with bi-exponential decay curves (with lifetimes fixed to the control conditions; see step 4.4 in protocol). The pixel colors indicate the estimated fractions F of syntaxin 4 in complex with VAMP3 (**Equation 3**). (**D**) Same as panel B, but for the bi-exponential fit. The convolution was performed with FIJI ImageJ using a custom-made macro (see **Table of Materials**). Please click here to view a larger version of this figure.
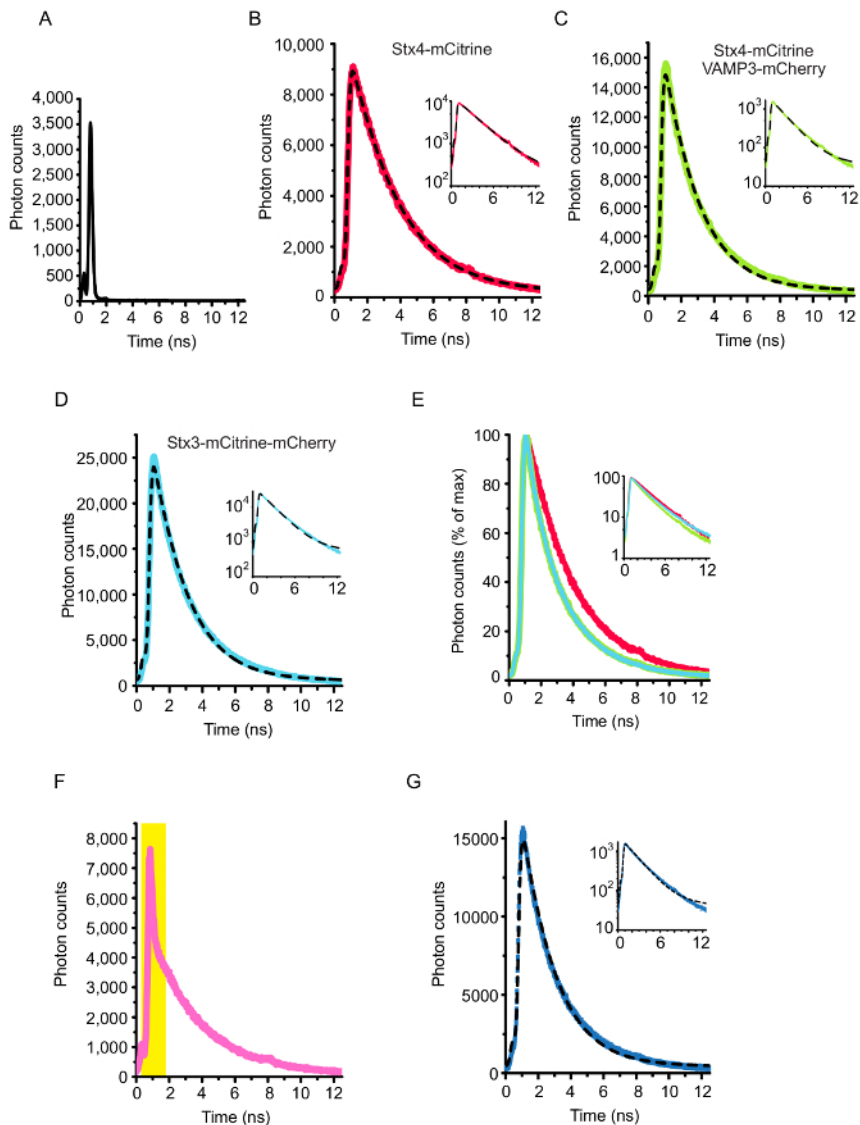
Figure 4: Whole cell FLIM analysis. (**A**) The instrument response function (IRF) of the setup. The IRF was measured using the back scattering on the glass-water interface (using a clean glass microscopy dish containing water). (**B-E**) Whole cell lifetime histograms for the cells shown in **Figure 2** and **Figure 3**. All photons present in the images were pooled. The curves were deconvoluted with the IRF and fitted with mono-exponential decay functions (**Equation 1**). For these cells, fluorescence lifetimes were obtained of 2.82 ns (cells expressing only syntaxin 4-mCitrine; donor only; panel B), 2.09 ns (cells co-expressing syntaxin 4-mCitrine with VAMP3-mCherry; panel C), and 2.08 ns (cells expressing the syntaxin 3-mCitrine-mCherry tandem construct; maximal expectable FRET control; panel D). The inlays show the same graphs, but now with logarithmic scaling of the y-axis. Panel E shows an overlay of the decay curves of panels B-D. (**F**) Example of a fluorescent lifetime histogram recorded too close to the surface of the microscope cover slip. This results in a large reflection peak (depicted by the yellow shaded area). (**G**) Same as panel C, but now fitting with a bi-exponential decay curve with lifetimes fixed to the control conditions (see step 4.4 in protocol). The amplitudes of the fast (A_1) and slow (A_2) components were 14.42 and 0.01, respectively, resulting in an estimated fraction of SNAREs in complex F of 0.99 (**Equation 3**). Please click here to view a larger version of this figure.

Supplementary File 1. Function file FLIM_convoluted_IRF Please click here to download this file.

Supplementary File 2. Function file FLIM_convoluted_IRF_biexp Please click here to download this file.

Discussion

This protocol demonstrates the use of FRET-FLIM for visualization of SNARE interactions between syntaxin 4 and VAMP3 in live HeLa cells. Syntaxin 4 is a Qa-SNARE protein predominantly locating at the plasma membrane where it mediates exocytosis[1,2,20,21]. VAMP3 is an R-SNARE which is mainly described to locate at recycling endosomal compartments and mediates trafficking to other endosomes as well as to the plasma membrane[1,2,20]. However, the FRET-FLIM assay can be readily adapted for studying other SNARE proteins. The only condition is that these SNAREs contain a C-terminal transmembrane helix, which is the case for most SNARE proteins by far[1,2]. In addition, the protocol described here can be adapted for visualization of SNARE complexes in any eukaryotic cell type, including plants and yeast. In this protocol, we used

the shortening of the fluorescence lifetime of the donor fluorophore as a measure of FRET. As a complementary approach, the lifetime of the acceptor fluorophore could be examined, because the sensitized emission causes a distinct rise phase which provides unambiguous proof that resonance energy transfer occurs.

Currently, the FRET-FLIM technique may be not able to visualize SNARE complexes in lysosomal compartments. For the syntaxin 3-mCitrine-mCherry tandem construct, the mCherry fluorescence can often be found more accumulated in a juxtanuclear area, which likely corresponds to lysosomal compartments, whereas the mCitrine signal is more abundant in the cellular periphery[5]. A similar juxtanuclear accumulation of mCherry compared to mCitrine was observed, when the same SNARE proteins fused to these fluorescent proteins were co-expressed[5]. Lysosomes are characterized by an extremely low pH (< 4) and a high activity of proteolytic enzymes. The juxtanuclear accumulation of mCherry is likely caused by a higher resistance of the mCherry fluorophore to lysosomal degradation compared to the mCitrine fluorophore. It is not due to pH-quenching of mCitrine, as juxtanuclear accumulation of mCherry also occurs upon fixation of the cells[5]. Thus, the FRET-FLIM technique underestimates the amount of FRET in the juxtanuclear (lysosomal) regions and this would require other fluorescent reporter proteins that survive the harsh conditions within the lumens of lysosomes.

FRET-FLIM in principle allows to obtain a (semi-)quantitative estimate of the fraction of SNAREs in complex[5]. As we explained in this protocol, this requires the fitting of the fluorescence lifetime histograms with double-exponential decay functions (**Equation 2**), where the amplitude of the fast component is proportional to the fraction of SNAREs in complex (**Equation 3**). However, such fitting with a two-component model is technically challenging. Fitting with multiple free fit parameters (two fluorescence lifetimes and two amplitudes) requires a very large number of photons, especially since the parameters will influence each other and small errors in the lifetime will affect the amplitudes and *vice versa*. To overcome these fitting problems, the fluorescence lifetimes of the slow component can be fixed to the lifetime of the donor only condition (*i.e.*, no FRET; only mCitrine present) and that of the fast component to the lifetime of the tandem construct (maximal expectable FRET). However, this should also be interpreted with care, because the fluorescence lifetimes may not be the same as in these control states, and could deviate because of multiple reasons (self-quenching, dipole orientation, variations in the microenvironment). Multiple SNARE complexes within close proximity (< 10 nm) can result in distance-dependent FRET, the same principle that allows FRET to be used as a "molecular ruler", but in this case, obscures the quantification of SNARE complexes. Moreover, a quantitative estimate is not always meaningful, because the labeled SNAREs compete with endogenous (unlabeled) SNAREs. As a consequence, the expression level of mCherry-labeled SNARE is a main determinant for the percentage FRET[5]. Because of all these caveats, it is recommended to fit the fluorescent lifetime histograms with a mono-exponential decay function (**Equation 1**). This has the advantage that it does not require any *a priori* knowledge of the lifetimes and the resulting apparent average fluorescent lifetime provides a solid measure for SNARE complexing[5].

Nevertheless, it is expected that quantitative FRET-FLIM imaging by two-component fitting models will have potent future applications. SNARE-encoding genes within the chromosome can be fused with fluorescent reporter proteins, for example by CRISPR/CAS9. This results in the fluorescent labeling of endogenous SNARE proteins, with endogenous protein levels and no background of unlabeled SNAREs, and thereby allows for a meaningful quantitative estimation of the fraction of SNARE complexes by FRET-FLIM. While the expression levels of endogenous SNAREs might be quite low and give relatively low fluorescent signals, it is expected that a sufficient number of photons can be obtained, especially for the whole cell FLIM (which requires only a few 1,000s of photons). Moreover, these FRET-FLIM measurements can be performed with more sensitive avalanche photodiode detectors which will also result in higher fluorescence signals and better photon statistics.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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