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TIRFM and pH-sensitive GFP-probes to evaluate neurotransmitter vesicle dynamics in SH-SY5Y neuroblastoma cells: cell imaging and data analysis

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Abstract:	Synaptic vesicles release neurotransmitters at chemical synapses through a dynamic cycle of fusion and retrieval. Monitoring synaptic activity in real time and dissecting the different steps of exo-endocytosis at the single-vesicle level are crucial for understanding synaptic functions in health and disease. Genetically-encoded pH-sensitive probes directly targeted to synaptic vesicles and Total Internal Reflection Fluorescence Microscopy (TIRFM) provide the spatio-temporal resolution necessary to follow vesicle dynamics. The evanescent field generated by total internal reflection can only excite fluorophores placed in a thin layer (<150 nm) above the glass cover on which cells adhere, exactly where the processes of exo-endocytosis take place. The resulting high-contrast images are ideally suited for vesicle tracking and quantitative analysis of fusion events. In this protocol, SH-SY5Y human neuroblastoma cells are proposed as a valuable model for studying neurotransmitter release at the single-vesicle level by TIRFM, because of their flat surface and the presence of dispersed vesicles. The methods for growing SH-SY5Y as adherent cells and for transfecting them with synapto-pHluorin are provided, as well as how to perform TIRFM and imaging. Finally, a strategy aiming to select, count, and analyze fusion events at whole-cell and single-vesicle levels is presented.

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UNIVERSITÀ DEGLI STUDI DI MILANO
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E BIOMOLECOLARI - DiSFeB

Milan, August 20, 2014

To Prof. Hannon Jane
Associated Editor
JoVE

Object: Cover Letter,

Dear Prof. Hannon Jane,

we carefully revised our manuscript entitled "**TIRFM and pH-sensitive GFP-based probes to evaluate neurotransmitter vesicle dynamics in the SH-SY5Y neuroblastoma cell line: cell imaging and data analysis**", by Daniele Federica, Di Cairano Eliana S., Moretti Stefania, Piccoli Giovanni, and Perego Carla.
The point-to-point response to criticisms raised in the e-mail letter of August 8th follows.
We trust that in the revised version the manuscript would be appropriate for JoVE.

Best regards,

Carla Perego

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TITLE:
TIRFM and pH-sensitive GFP-probes to evaluate neurotransmitter vesicle dynamics in SH-SY5Y neuroblastoma cells: cell imaging and data analysis.

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KEYWORDS:
Synaptic vesicles, neurotransmission, Total Internal Reflection Fluorescence Microscopy, pHluorin, neuroblastoma cells

SHORT ABSTRACT:
This paper provides a method for investigating neurotransmitter vesicle dynamics in

neuroblastoma cells, using a synaptobrevin2-pHluorin construct and Total Internal Reflection Fluorescence Microscopy. The strategy developed for image processing and data analysis is also reported.

LONG ABSTRACT:

Synaptic vesicles release neurotransmitters at chemical synapses through a dynamic cycle of fusion and retrieval. Monitoring synaptic activity in *real time* and dissecting the different steps of exo-endocytosis at the single-vesicle level are crucial for understanding synaptic functions in health and disease.

Genetically-encoded pH-sensitive probes directly targeted to synaptic vesicles and Total Internal Reflection Fluorescence Microscopy (TIRFM) provide the spatio-temporal resolution necessary to follow vesicle dynamics. The evanescent field generated by total internal reflection can only excite fluorophores placed in a thin layer (<150 nm) above the glass cover on which cells adhere, exactly where the processes of exo-endocytosis take place. The resulting high-contrast images are ideally suited for vesicle tracking and quantitative analysis of fusion events.

In this protocol, SH-SY5Y human neuroblastoma cells are proposed as a valuable model for studying neurotransmitter release at the single-vesicle level by TIRFM, because of their flat surface and the presence of dispersed vesicles. The methods for growing SH-SY5Y as adherent cells and for transfecting them with synapto-pHluorin are provided, as well as how to perform TIRFM and imaging. Finally, a strategy aiming to select, count, and analyze fusion events at whole-cell and single-vesicle levels is presented.

To validate the imaging procedure and data analysis approach, the dynamics of pHluorin-tagged vesicles have been analyzed under resting and stimulated (depolarizing potassium concentrations) conditions. Membrane depolarization increases the frequency of fusion events and causes a parallel raise of the net fluorescence signal recorded in whole cell. Single-vesicle analysis reveals modifications of fusion-event behavior (increased peak height and width). These data suggest that potassium depolarization not only induces a massive neurotransmitter release but also modifies the mechanism of vesicle fusion and recycling.

With the appropriate fluorescent probe, this technique can be employed in different cellular systems to dissect the mechanisms of constitutive and stimulated secretion.

INTRODUCTION:

Chemical synaptic transmission between neurons is a major mechanism of communication in the nervous system. It relies on the release of neurotransmitters through a dynamic cycle of vesicle fusion and retrieval at the presynaptic site. Many of the proteins involved in vesicle dynamics have been identified; however, their specific contribution to the phenomenon remains to be clarified[1].

Our understanding is partly limited by the fact that the most widely used assays for exo/endocytosis are not always the most appropriate. Several studies related to vesicle fusion

and dynamics rely on electrophysiological techniques. This technique provides an optimal temporal resolution and is excellent for investigating the initial fusion of vesicles to the plasma membrane but is unable to detect many of the underlying molecular events that support presynaptic function. Electron microscopy, on the other side, provides the finest morphological description of each singular step, but the dynamic aspect of the event cannot be captured, because samples must be fixed in order to be analyzed.

The advent of new optical recording techniques[2,3], in combination with advances in fluorescent molecular probes development[4-6], enable the visualization of exocytic processes *in live* cells, thus providing new levels of information about the synaptic structure and function.

Initial studies exploited activity-dependent styryl dyes (FM1–43 and related organic dyes)[7,8]. State-of-the-art imaging techniques employ pH-sensitive variants of the Green Fluorescent Protein (GFP) (pHluorin) tethered to luminal vesicles proteins[9]. These probes are normally switched off when present in the vesicles because of the low luminal pH. After fusion with the plasma membrane, the vesicle interior is exposed to the neutral extracellular space, the pH abruptly increases, relieves the proton-dependent quenching of pHluorin and the fluorescent signal rapidly appears. As the change in pHluorin is faster than the fusion event, by monitoring fluorescence increases, vesicle fusion with the membrane can be measured and analyzed. Because surface pHluorin-tagged molecules are endocytosed, the fluorescence signal subsequently returns to basal level, therefore the same construct may be used also to monitor vesicle recycling[9].

While the vesicle-tagged pH-sensor ensures the visualization only of those vesicles that really fuse with the plasma membrane, imaging at high spatial and temporal resolution is required to describe in details the steps involved in the exo/endocytosis processes. The optical technique that provides the necessary spatio-temporal resolution is total internal reflection fluorescence microscopy (TIRFM), an application of fluorescence microscopy.

Total internal reflection occurs at the interface between the glass cover-slip and the sample. When the light path reaches the glass cover-slip with an incident angle larger than the critical angle, the excitation light is not transmitted into the sample but is completely reflected back. Under these conditions, an evanescent light wave forms at the interface and propagates in the medium with less optical density (the sample). As the intensity of the evanescent field decays exponentially with distance from the interface (with a penetration depth of about 100 nm) only the fluorophores in closest proximity to the cover-slip can be excited while those further away from the boundary are not. In cells transfected with GFP-constructs, this depth corresponds to proteins expressed on the plasma membrane or in vesicular structures approaching it. As fluorophores in the cell interior cannot be excited, the background fluorescence is minimized, and an image with a very high signal/background ratio is formed[11].

Several characteristics make TIRFM the technique of choice for monitoring vesicles dynamics. The perfect contrast and the high signal-to-noise-ratio allow the detection of very low signals deriving from single vesicles. Chip-based image acquisition in each frame provides the temporal

resolution necessary to detect highly dynamic processes. Finally, the minimal exposure of cells to light at any other plane in the sample strongly reduces phototoxicity and enables long lasting time-lapse recording[12].

Data analysis remains the most challenging and crucial aspect of this technique.. The simplest way to monitor vesicle fusion is to measure the accumulation of reporter fluorescent proteins at the cell surface, over time[13]. As fusion increases, net fluorescence signal increases as well. However, this method may underestimate the process, particularly in large cells and in resting conditions, because endocytosis and photobleaching processes offset the increase in fluorescence intensity due to vesicle exocytosis. An alternative method is to follow each single fusion event[14]. This latter method is very sensitive and can reveal important details about the fusion mechanisms. However, it requires the manual selection of single events, because completely automated procedures to follow vesicles and to register the fluctuation of their fluorescent signals are not always available. Observation of vesicle dynamics requires sampling cells at high frequency. This generates a large amount of data that can hardly be analyzed manually.

The proposal of this paper is to optimize the TIRFM imaging technique for monitoring the basal and stimulated neurotransmitter release in the SH-5YSY neuroblastoma cell line, and to describe, step-by-step, a procedure developed in the laboratory to analyze data, both at whole-cell and single-vesicle levels.

PROTOCOL:

1. Cell culture and transfection

1.1) SH-SY5Y cell culture

Note: The experiments have been performed using the human neuroblastoma SH-**SY5Y** (ATCC# CRL-2266)[15]. SH-SY5Y cells grow as a mixture of floating clusters and adherent cells. Follow the instructions reported in the protocol (cell density, splitting ratio, etc.) to have cells that grow firmly attached to glass cover, which is crucial for TIRFM.

1.1.1) Before starting, under the laminar flow biosafety cabinet, make the opportune volume of sterile phosphate buffer saline solution (PBS) and culture medium.

1.1.1.1) Make 50 ml of PBS with concentrations of 150 mM NaCl, 24 mM phosphate buffer, pH 7.4. Filter the solution.

1.1.1.2) Make 50 ml of cell medium from Dulbecco's modified Eagle medium (DMEM) with high glucose, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and sodium pyruvate (1 mM). Filter the solution.

1.1.2) Remove complete growth medium and wash the cells with 3 ml of PBS.

1.1.3) Incubate cells with 2 ml of 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (for 6 cm Petri dish) for 5 minutes at 37 °C, 5% CO_2 and detach cells using pipette.

1.1.4) Inactivate trypsin by adding 2 ml of DMEM, and collect cells by centrifugation at 300 x g for 5 minutes.

1.1.5) Remove the supernatant, add 1 ml of DMEM to the pellet and pipette the solution up and down sufficiently to disperse cells into a single cell suspension.

1.1.6) Split them 1:4 in a new 6 cm diameter Petri dish containing 3 ml of complete medium. Maintain cells in culture in 6 cm diameter Petri dishes, at 37 °C in a 5% CO_2 incubator. Sub-culture once a week or when they have covered 80-90% of the surface area.

1.2) **SH-SY5Y cell plating for imaging**

1.2.1) For TIRFM experiments, plate cells onto glass covers. Employ glass covers with 0.17 ± 0.005 mm thickness and a 1.5255 ± 0.00015 refractive index. Before starting, prepare the glass coverslips as follow:

1.2.1.1) Clean glass covers with 90% ethanol, overnight.

1.2.1.2) Rinse them thoroughly in distilled water (three changes of distilled water). Dry glass covers in a drying oven.

1.2.1.3) Place covers in glass Petri dishes and sterilize in a preheated oven at 200 °C for 3 hours.

1.2.2) The day before transfection, place each coverslip in a 3.5 cm Petri dish, add 1 ml of culture medium and incubate at 37 °C in a 5% CO_2 incubator.

1.2.3) Trypsinize cells as described in 1.1.3-1.1.5, suspend the cell pellet in 1 ml of complete medium and count. Calculate the correct volume of cell suspension to add to each Petri dish to yield $3x10^5$ cells/well. This density is required for optimal cell growth and efficient transfection. Incubate at 37 °C in a 5% CO_2 incubator overnight.

1.3) **SH-SY5Y transfection by polyethylenimine (PEI)**

Note: To visualize synaptic vesicles dynamics, pCB6 vector containing synapto-pHluorin has been used. The synapto-pHluorin has been generated by in frame fusion of a pH-sensitive variant of the green fluorescent protein (GFP)[16] and the vesicular membrane protein synaptobrevin 2. The construct has been extensively employed to investigate synaptic vesicle

properties within neurons[9].

1.3.1) Before starting transfection, make 10 ml of the following solutions. Keep the solutions as maximal as 1 month.

1.3.1.1) Make a 150 mM NaCl solution. Adjust to pH 5.5 with 0.01 N HCl.

1.3.1.2) Make a PEI solution at 10% Polyethylenimine (PEI) (25 kDa linear) in 150 mM NaCl solution. The pH of solution rises to 8.8. Adjust pH to 7.8 with 0.01 N HCl.

1.3.2) Twenty-four hours after plating, remove the medium and refresh with 1.5 ml of complete medium. Keep the cells at 37 °C, in a 5% CO_2 incubator.

1.3.3) Under the laminar flow biosafety cabinet, in a 1.5 ml microfuge tube, add 3 µg of plasmid DNA to 25 µl of 150 mM NaCl solution and 100 µl of PEI solution per 3.5 cm Petri dish.

1.3.4) Vortex for 10 sec, then incubate the DNA/PEI mixture for 30 min at room temperature.

1.3.5) Carefully add the DNA/PEI mixture to the Petri dish containing coverslips with cells and gently shake to equally distribute the reagent in the Petri dish.

1.3.6) After 4 hours change the medium and incubate the cells overnight at 37 °C in a 5% CO_2 incubator. Perform imaging experiments 24-48 hours after transfection.

2. **Cell imaging by Total Internal Reflection Fluorescence Microscopy (TIRFM)**

2.1) Imaging set-up

2.1.1) Perform TIRF imaging with the set-up described in **Figure 1**. It comprises a motorized inverted microscope (**Figure 1, inset A**), the laser source (**Figure 1, inset B**) and the TIRF-slider (**Figure 1, inset C**). Reach TIRFM illumination through a high numerical aperture (NA 1.45 Alpha Plan-Fluar) 100 × oil, immersion objective.

2.1.2) For TIRFM illumination, employ a multi-line (458/488/514 nm) 100 mW argon-ion laser. Using a mono mode fiber, introduce the linearly polarized laser light into the beam path, via the TIRF slider. Insert the TIRF slider into the luminous field diaphragm plane of the reflected-light beam path.

2.1.2.1) For wide field illumination, connect the microscope to a conventional mercury short-arc lamp HBO white light. A polarization-maintaining double prism in the slider ensures the simultaneous combination of TIRF illumination and white light.

2.1.3) Filter the laser light with an excitation filter (band width 488/10 nm) mounted on a filter

wheel, introduced into the laser path. Employ a high speed, software-controlled, shutter to allow fast control of laser illumination. For pHluorin analysis, mount a band pass 525/50 nm emission filter. Capture digital images (512 × 512 pixels) on a cooled Fast CCD camera with the Image ProPlus software.

2.2) **Achieving TIRF illumination (Figure 2)**

2.2.1) Turn on the lasers, the computer, the camera, the filter wheel, and the shutter controllers; then, wait 20 min before starting the experiment as the lasers need to warm up and stabilize.

2.2.2) Before imaging, make the opportune volume of the following solutions.

2.2.2.1) Make 50 ml of Krebs (KRH) solution at 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (buffered to pH 7.4), 2 mM $CaCl_2$, and 6 mM glucose.

2.2.2.2) Make 10 ml of KCl-KRH solution (pH 7.4) at 80 mM NaCl, 50 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 25 mM HEPES (buffered to pH 7.4), 2 mM $CaCl_2$, and 6 mM glucose.

2.2.3) Remove the glass cover with transfected cells and insert it in the appropriate imaging chamber. Assemble the chamber and add 500 µl of KRH solution in the center of the glass.

2.2.4) Add oil over the objective. Place the imaging chamber on the stage of the microscope and position the objective under the glass coverslip. Position the safe cover over the sample.

2.2.5) In epifluorescence mode, focus on the coverslip (upper surface) and choose transfected cells placed in the chamber center. Select cells whose fluorescent signal can be clearly recorded using an exposure time below 80 msec.

2.2.6) Under software control, switch to TIRF illumination *in live* mode.

2.2.7) To set the TIRF configuration, check the position of beam that emerges out of the objective, on the sample cover (**Figure 2B**). When the beam is positioned in the center of the objective lens (**Figure 2A, left**), a spot is visible in the center of the TIRF sample cover (**Figure 2B, left**) and the cell is imaged in epifluorescence mode (several focus planes, high background fluorescence) (**Figure 2C, left**).

2.2.8) To reach the critical angle, move the focused spot in the Y direction (forward or backward) (**Figure 2B, center**) using the angle adjustment screw on the TIRF slider (**Figure 1, C**). When the beam converges on the sample plane at an angle larger than the critical angle (**Figure 2A, rig**ht), the spot disappears and a straight, thin, focused line is evident in the middle of the sample cover (**Figure 2B, right**).

2.2.9) To fine-tune the TIRF angle use the cell sample (**Figure 2C**). Watch the fluorescence image on the video, at this stage, an epifluorescence-like image is still visible. Gently, move the screw until TIRF condition is achieved: only one optical plane of the cell is in focus (*i.e.,* the plasma membrane in contact with the cover-slip), this results in a flat image with high contrast (**Figure 2C, right**).

2.3 Sample imaging

2.3.1) Set the single-channel time-lapse experiment. To minimize photobleaching, capture the image using low exposure time and high gain. Appropriate exposure times are between 40-80 msec. Acquire images at 1 Hz sampling frequency. Vesicle kinetics may be better appreciated sampling at higher frequency (10 Hz). The regular time of observation is usually 2 minutes.

2.3.2) Add 500 µl of KRH solution and record cells in TIRFM mode. This is the resting condition. Save the time sequential images.

2.3.3) Focus on the same cell and record under the same conditions of resting (laser power, time exposure, frame number). After five frames, add 500 µl of KCl-KRH solution and keep KCl in the chamber. This is the stimulated condition; save the time sequential images.

3. Image analysis and data processing

Note: To analyze images, macros have been developed in the lab, based on existing functions of the image analysis software; similar macros are available online (URL provided in “table of materials and equipments”).

3.1) Fluorescence intensity quantification

3.1.1) Use a “Sequence fluorescence intensity” macro for fluorescence intensity quantification in a region of interest (ROI) of the image, over the course of the movie.

3.1.2) Open the time-sequential images. Go to the macro menu and select "Sequence fluorescence intensity". In the "Analysis" window appears “select the ROI”.

3.1.3) Choose one of the selection tools in the menu to create the ROI. Place 3 ROIs in regions of the cell membrane without spots (background ROI). Employ this “background ROI” to evaluate the photobleaching and to set the threshold for fusion event analysis (**Figure 3A**).

3.1.4) With the ROIs selected, click "OK". Automatically, calculate the average fluorescence intensity of each ROI over the course of the movie. Export data to a spreadsheet program for further analysis.

3.2) Photobleaching correction and threshold determination (Figure 3B)

3.2.1) To evaluate the photobleaching, open the fluorescence intensity rows “background ROIs”, (**Figure 3Ba**). Normalize the fluorescence intensity values in each frame to the initial intensity value (F0) (F/F0) (**Figure 3Bb**). Average the values.

3.2.2) Highlight the average data and create a line plot using the chart menu options.

3.2.3) From the data analysis menu, select “trendline” to open the plot analysis dialog. Select the type of regression. Set “exponential” regression. Then select “display equation on chart”. In the graph window, the exponential equation appears and the parameter values are automatically assigned, (**Figure 3Bc**).

3.2.4) Apply the exponential correction to the intensity values in each frame as follow:
Fn (corrected) = Fn/exp(-n*a)
Fn = experimental fluorescence intensity measured at frame n; n = number of frames; a = bleaching factor (constant that expresses the rate of intensity loss due to photobleaching), (**Figure 3Bd**).

3.2.5) To set the threshold, open a normalized and corrected “background ROI”, calculate the average fluorescence signal and its standard deviation (SD). The average value plus 3 SD represents the threshold (**Figure 3Be**). Use this threshold for data analysis.

3. 3) Selection of fusion events using a semiautomatic procedure

3.3.1) Open the time-sequential images with image analysis software. Apply a Gaussian filter to the active image sequence.

3.3.2) Analyze images using the tool “count objects” or a macro which allows the selection of an object whose pixels have average fluorescence intensity within a defined range. Set the intensity range manually, using the threshold function (go to the bar menu, set measure → threshold to highlight the area of interest). An adequate threshold is 30% over the local fluorescent background signal.

3.3.3) Apply a macro “Filters objects” to select only objects meeting the following criteria:

3.3.3.1) Apply ranges option (min and max inclusive) for aspect. **Aspect** reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object. Aspect is always ≥1. Adequate values are min=1, max=3.

3.3.3.2) Apply ranges for diameter. **Diameter** reports the average length of the diameters measured at two degree intervals joining two outline points and passing through the centroid of the object. Set the range in pixels (or in µm, if using a calibrated system).

3.3.3.3) Define the optimal range in preliminary experiments: select manually the spots of

interest and then measure their diameter using the plot profile function.

3.3.4) Select “display objects”: selected objects will appear superimposed to the TIRFM image (**Figure 4B**).

3.3.5) Include in the analysis only those spots that show a short (one to three frames) transient increase in fluorescence intensity, immediately followed by a marked loss of signal (transient spots). Employ the circular selection to create a ROI approximately one-spot diameter radially around the selected vesicle/spots (experimental ROIs). Perform this step manually.

3.3.6) With the ROIs selected, calculate the average fluorescence intensity of each ROI over the course of the movie.

3.4) Data analysis (Figure 3C-D)

3.4.1) Export the time-course of the fluorescence changes measured in each “experimental ROI” to a spreadsheet; (**Figure 3Da**). Normalize the intensity value in each frame to the initial fluorescence intensity (F/F0), (**Figure 3Db**).

3.4.2) Apply the exponential correction to the intensity values in each frame as reported in 3.2.4, (**Figure 3Dc**).

3.4.3) To calculate the total number of fusion events (peak number), the time each fusion occurs (peak width) and the amplitude of fluorescent peak (peak height and AUC) apply logical functions using spreadsheet or math packages. An example of fusion event analysis using logical formulas is reported in Panels 3De and 3Df.

3.4.4) Assume the increase of fluorescence intensity exceeding the threshold (average background fluorescence intensity±3SD) as vesicle fusion to the plasma membrane and the resulting peak as a fusion event.

3.4.5) Calculate the peak width as difference between the last and the first x value of each peak. Multiply this value for 1/(sampling frequency). Consider this value as the time of vesicle fusion and adhesion at the plasma membrane before vesicle re-acidification and recycling, (Figure 3Df).

3.4.6) Calculate the whole-cell AUC as a sum of values over threshold. Consider this value as net fluorescent change during the recording time due to the spontaneous (resting) or evoked (stimulated) synaptic activity.

3.4.7) Calculate the peak height as the difference between the maximal y value of each peak and the threshold. Consider this value as indicative of the fusion type (single *vs.* simultaneous/sequential fusion or transient *vs.* full fusion).

REPRESENTATIVE RESULTS:

The TIRF imaging and data analysis procedures described are designed to study vesicles dynamics in cellular systems. This technique can be used to determine the effects of signaling molecules and drugs on fusion events and neurotransmitter vesicle dynamics[17]. Using GFP-tagged plasma membrane proteins, the TIRFM analysis has been employed to characterize the constitutive trafficking of GFP-tagged glutamate transporters in glial and epithelial cells[18,19].

To validate the imaging procedure and data analysis strategy reported, fusion events have been recorded under basal and stimulated conditions (potassium-induced depolarization), in SH-SY5Y neuroblastoma cells transfected with synapto-pHluorin. (**Video 1, 2**, respectively). (**Video 1, 2**, respectively). Two different analyses have been performed: whole-cell (**Figure 4**) and single-vesicle analyses (**Figure 5**).

Whole cell analysis measures the total number of fusion events in the cell and the resulting net fluorescence changes induced by stimulation. In **Figure 4,** synapto-pHluorin transfected cells are recorded under resting and stimulated conditions (KCl stimulation), using the same experimental protocol (time exposure, laser power, etc). **Panel 4A** shows that synapto-pHluorin accumulates in fluorescent puncta scattered on the cell membrane. As described in literature, a faint fluorescent signal is also present at the plasma membrane[9]; this signal is useful to identify cells to be imaged. In **Panel 4B,** spots selected by the automatic procedure described in the paper (paragraph 3.3) are superimposed to the TIRFM image reported in Panel 4A. **Panel 4C** shows the normalized fluorescent intensity profiles of selected spots under resting conditions. These profiles reveal the presence of individual peaks of similar fluorescent intensity that come out at various times during the recording and probably correspond to vesicles that occasionally fuse with the membrane. **Panel 4D** shows the effects of KCl stimulation. As expected, depolarization with 25 mM KCl elicits a prompt response and several very bright fluorescent puncta appear at the cell membrane (**Video 2**). These puncta correspond to the 'readily releasable' pool of synaptic vesicles present beneath the plasma membrane. The time course analysis of fluorescent changes measured in correspondence of individual spots indicates the presence of peaks, of variable fluorescence intensity, that appear suddenly after application of the secretory stimulus (**Panels 4D and 4F)**. Results of whole cell analysis during the time of recording are reported in Panels E-H. KCl stimulation causes a rapid marked increase in the number of fusion events (2.5 fold increase over resting conditions) (**Panels 4E-F**) and in the resulting fluorescence intensity changes (9.3 fold increase over resting conditions) (**Panels 4G-H**), thus indicating massive neurotransmitter release.

Single-peak analysis allows the characterization of single-fusion events (**Figure 5**). **Panel 5A** shows the sequential images of a representative "experimental ROI" recorded under resting conditions. The particular highlights a synapto-pHluorin labeled vesicle which fuses with the membrane under the TIRF zone. After two frames, the fluorescent signal disappears, indicating probable vesicle retrieval and re-acidification. The normalized fluorescence profile of the region of interest (**Panel 5B)** measures an increase in the fluorescent signal in correspondence of the spot appearance in the TIRF zone. Conversely, the fluorescence returns to basal level after spot

disappearance (single peak average width 1.91 ± 0.32 seconds; average peak height 0.042 ± 0.005 normalized fluorescence intensity). **Panel 5C** and 5D show the sequential images of an "experimental ROI" recorded under KCl stimulation and the corresponding normalized fluorescence intensity profiles. Note the increase in the movement of vesicles in and out the TIRF zone after KCl depolarization.

Forty fusion events have been selected and analyzed in resting and stimulated conditions. The following parameters have been measured: average peak AUC, peak width and height. Peak width specifies the time of vesicle fusion, attachment and endocytosis before re-acidification and recycling, **Panel 5F**. Peak height measures the fluorescence intensity changes induced by vesicle fusion, **Panel 5G**. Changes in these parameters are indicative of different exocytosis mechanisms. Single-peak analysis reveals that KCl depolarization modifies the mode of vesicle fusion to the plasma membrane. Indeed, increase in the average peak area (3.8 ± 0.2 fold increase over resting conditions, $P < 0.01$ by paired t-test, **Figure 5E**), peak height (2.75 ± 0.03 fold increase, $P < 0.01$ by paired t-test, **Figure 5F**) and width (2.6 ± 0.5 fold increase, $P < 0.05$ by paired t-test. **Figure 5G**) are detected under stimulated conditions. Several explanations can be envisaged for these results. A possibility is that KCl depolarization causes the simultaneous and/or sequential fusion of vesicles in a constrained region of the cells. An alternative explanation is that strong depolarization favors full fusion versus transient fusion. Under basal conditions, the prevailing mechanism is a transient fusion: a fusion pore forms, the pH in the vesicle increases and the fluorescent signal appears, but the pore immediately closes, thus allowing rapid re-acidification and recycling. Under stimulated conditions, the vesicle completely fuses with the plasma membrane, the peak height and width increase as recapture of membrane vesicle components, re-acidification and recycling may require a longer period. A similar result has been recently obtained analyzing synaptic-like microvesicle exocytosis in endocrine β-cells[20].

Figure 1: TIRF microscope set up.
Schematic view and image (inset) of the TIRF microscope system. The set up comprises the Axio Observer Z1 motorized inverted microscope (A), a multi-line 100 mW Argon-ion laser (B) and a TIRF-slider (C). The laser light (green line) and white light (yellow line) are shown. Cells are imaged using a 100 × oil immersion objective. Digital images are captured on a cooled RetigaSRV Fast CCD camera. The reflector module, the emission (488/10 nm) and the excitation (band pass 525/50 nm) filters, the collimator (L1) and focusing (L2) lens are indicated.

Figure 2: Getting TIRFM configuration.
A) Schematic cartoon illustrating the objective, the cover-slip, the sample and the position of the laser beam (blue line). *Left,* the excitation beam travels directly through the cover-slip–sample interface. The sample is excited as in epifluorescence mode. *Center*, the excitation beam forms with the sample an incident angle lower than the critical angle, the light illuminates the sample at a variable angle. *Right*, the excitation beam forms an incident angle greater than the critical angle, the light is completed reflected back into the objective lens, and an evanescent field propagates in the sample. B) Cartoon showing the sample cover and the position of the excitation beam (blue circle), that emerges out of the objective, during the transition from

epifluorescence (*left*) toward TIRF illumination (*right*). C) epifluorescence (*left)* and TIRFM (*right)* images of synapto-pHluorin fluorescence in a live SH-SY5Y cell. Scale bar: 10 µm.

Figure 3: Data processing and analysis

A) TIRFM image of synapto-pHluorin fluorescence in a live SH-SY5Y cell. The green square indicates a representative "background ROI". Scale bar 10 µm. B) Proposed workflow for background ROI. From top to bottom: a. time course of fluorescence intensity changes measured in the background ROI; b. normalization of fluorescence changes to the initial fluorescence value (F/F0); c. application of the exponential regression; d. correction for photobleaching, e. threshold evaluation (transparent gray square). C) TIRFM image of synapto-pHluorin fluorescence in a live SH-SY5Y cell. The white square indicates a representative "experimental ROI". Scale bar 10 µm. D) Proposed workflow for experimental ROI. From top to bottom: a. time course of the fluorescence intensity changes; b. data normalization to the initial fluorescence value (F/F0); d. photobleaching correction; d-e. application of logical functions to detect peak number, AUC, width and height.

Figure 4: Whole cell analysis

A) TIRFM image of synapto-pHluorin fluorescence in a live SH-SY5Y cell. Cells are recorded under resting and stimulated (25 mM KCl application) conditions (sampled at 1 Hz). Scale bar: 10 µm. B) spots identified by the automatic procedure are shown superimposed (green color) on the TIRFM image. C) Normalized fluorescence intensity profiles (F/F0) of spots selected by the automatic procedure in the whole cell under resting conditions. D) Normalized fluorescence intensity profiles (F/F0) of selected spots under stimulated conditions. The bar over the traces indicates KCl application. E-H) Number of events and fluorescence intensity changes recorded in the whole cell under resting (blue) and stimulated (red) conditions. E) Histograms showing the total number of fusion events recorded in the cell. F) Temporal distribution of fusion events. G) Histograms representing changes (Total AUC) in pHluorin fluorescence intensity occurring in the whole-cell. H) Curves showing the cumulative pHluorin fluorescence intensity changes as a function of time.

Figure 5: Single-vesicle analysis

A) SH-SY5Y cells expressing synapto-pHluorin are imaged at 1 Hz, under resting conditions. Representative TIRFM sequential images (every 2 seconds) of a ROI showing a synapto-pHluorin labeled vesicle. ROI= 40x35 pixels. B) Normalized fluorescence profile (F/F0) of the ROI shown in A. The black asterisk indicates a fusion event, the threshold line is shown. C) The same cell is recorded under stimulated conditions (25 mM KCl), representative TIRFM sequential images of a ROI are shown. KCl application is indicated by the yellow asterisk. D) The normalized fluorescence profile of the region shown in C highlights the arrival (in) and disappearance (out) of vesicles. Black asterisks indicate fusion events, the threshold line is shown. E-G) properties of single-vesicle events recorded under resting (blue bar) and stimulated (red bar) conditions. n=40 fusion events. E) *Left*, peak area (AUC) is indicated by light blue; *right*, histograms of average peak areas; **p<0.01. F) *Left*, peak height (h) is indicated by a double-headed arrow; *center*, histograms of the average peak height; **p<0.01; *right*, peak height specifies the fusion mechanism. The star in the cartoon indicates synapto-pHluorin. G) *Left,* peak width is indicated

by a double-headed arrow; *center*, histograms of the average peak width; *p<0.05; *right*, peak width specifies the time of vesicle exocytosis, attachment and endocytosis. The star color is green when synapto-pHluorin fluorescence is visible and gray when it is switched-off.

Video 1. SH-SY5Y cells expressing synapto-pHluorin are recorded under resting conditions (sampled at 1 Hz).

Video 2. SH-SY5Y cells expressing synapto-pHluorin are recorded under stimulated conditions (sampled at 1 Hz). KCl perfusion is indicated.

DISCUSSION:

This paper presents a protocol to image and analyze vesicles dynamics in secreting cells, using fluorescent cDNA-encoded vectors and TIRFM. Key elements of successful imaging by TIRFM are the selection of the cellular model and cell transfection with genetically-encoded optical indicators of vesicle release and recycling.

TIRFM is ideally suited for cells growing adherent to a glass cover and sufficiently flat to allow stable visualization of membranes and fusion events. Vesicles should ideally be dispersed in the cell so that their trafficking, fusion and endocytosis can be imaged and quantified at single-vesicle level. Unfortunately, neurons do not meet these criteria: they have irregular shapes, neurites which frequently cross over each other, and vesicles fusion is prevalently concentrated in small regions (active zones). For these regions is very difficult to study vesicle dynamics by TIRFM in primary cultures of neurons.

We demonstrate for the first time that the SH-SY5Y human neuroblastoma cell line can be a valuable alternative model to investigate neurotransmitter release under resting and stimulated conditions, by TIRFM. This cell line has a neuronal phenotype with a cellular body and thin processes, possesses enzymes for neurotransmitter synthesis, proteins of the synaptic machinery, and a regulated secretion. Furthermore, it can be differentiated into a functionally mature neuronal phenotype in the presence of various agents, including retinoic acid, phorbol esters, and dibutyryl cyclic AMP[21,22]. Cells are sufficiently flat to allow stable visualization of membranes and fusion events in TIRFM mode (particularly in the cell body) and vesicles are relatively dispersed. Finally, cells can be easily transfected with plasmid encoding GFP-labeled proteins or pH-sensitive protein tags using different transfection reagents. In this protocol, PEI has been used to transfect the cells. This reagent constitutes the basis of most commercially available transfection agents and alone acts as a very cost-effective transfection vector. A 20% efficiency of transfection is expected using the above reported protocol which is adequate for single cell imaging.

While the availability of different transfection reagents and procedures makes transfection almost a standard procedure in SH-SY5Y and even in primary neuronal cultures, care must be taken when recording, analyzing and interpreting TIRFM data. TIRFM facilitates the collection of information regarding processes that occur at or near the membrane in living cells, and enables the analysis of individual molecular events through detection of changes in the fluorescent

signal derived from tagged proteins that move in or out the evanescent filed. However, several factors can modify the fluorescent signals in this zone, without necessarily implying exo/endocytic events, and this must be taken into consideration when recording and analyzing data. Among these are morphological changes in the cell, particularly those concerning the plane in focus under the evanescent field and fluorophore modifications during recording.

Morphological Changes

The high resolution of the TIRF technique relies on the excitation of fluorophores within the evanescent field, with the depth of 100 nm from the glass interface[11]. This is a very thin zone and imperceptible morphological modifications are expected to change the cell plane in focus. This particularly applies to neurons and cells that present several processes and exhibit pronounced ruffling. In these cells, the membrane area in contact with the cover-slip during recording is irregular and can rapidly change, thus causing inaccurate evaluation of exocytosis. For this reason, whenever possible, it is important to select the cellular model of investigation. To limit cell movements can be helpful to coat glass-covers with extracellular matrix proteins or poly-l-lysine. However, one must keep in mind that these substrates may modify cell behavior and vesicle dynamics.

Other possible sources of morphological modifications during recording are cell stimulation, addition of solutions, and temperature changes. Stimuli able to induce massive vesicles release (*i.e.,* KCl depolarization) often cause cell shrinkage which obviously modifies the cell surface under the TIRF zone. It is therefore important to select accurately the type, concentration, and application time of the stimulus in preliminary experiments.

The simple introduction of solutions into the bath with a pipette, independently of the composition, may cause modification of cell morphology by shear stress. To solve this artifact, add medium preferably using a perfusion system, possibly connected with a vacuum pump to reduce noise.

Cell morphology and function are extremely sensitive to temperature variations due to experimental environment, medium addition and intense laser illumination. The temperature control in live-cell imaging is normally achieved using incubators; small (stage-top) and large (chamber) incubators are available. The former are particularly handy and well suited for the observation of cell cultures, the latter guarantee a constant temperature of all devices inside the incubator, including a large part of the microscope, thus minimizing the focus drift resulting from temperature gradient. In the absence of a temperature regulatory system, it is critical to equilibrate cells, live-cell imaging chamber, and solutions to room temperature and to avoid long-term imaging experiments.

Fluorophore

Alteration of fluorescent signals may also be due to modification of the fluorophore during recording. The most important is photobleaching[23]. Photobleaching is the photon-induced decomposition of a fluorophore. It generally causes a permanent loss of fluorescence and dimming of the observed sample over time. In TIRFM, only fluorophores closed to the origin of

the evanescent field can be photobleached and GFP-tagged membrane proteins are photobleached because they reside in this field. The prevention of the fading of fluorescence emission intensity is very important to obtain high-quality images, and obligatory for quantitative fluorescence microscopy. With a reasonable approximation, for a given molecule in a constant environment, photobleaching depends on the time and the cycle of exposure to the excitation source. In many instances, photobleaching follows a simple exponential decay function, which makes its assessment and its correction easier by performing control recordings[23]. Different correction formulas/macros are available online (see Table of materials and equipments); in the protocol a simple exponential function has been used.

There are several strategies to overcome photobleaching. A good strategy is to prevent photobleaching at the source, for example, using fluorophores with high photostability. Unfortunately, right now, the choice of DNA-encoded probes is still limited. In this case, loss of activity caused by photobleaching can be minimized during imaging acquisition, optimizing time-span of light exposure, the photon energy of the input light and the frequency of sampling.

When using pH-sensitive probes, a further source of fluorophore modification during recording is the pH shift in the medium. The liquid volume of the recording chamber is usually very low and drug application, cell activity and metabolism may modify the pH of the medium, particularly in the tiny volume between the cell and the surface of the coverslip. This in turn, changes the pHluorin fluorescent signal, thus causing over/under-estimate vesicle release. For example, strong stimulations may lead to a calcium-dependent acidification of the cytosol and mirrored alkalization in the extracellular space, thus resulting in an exaggerated increase in the fluorescent signal[24].

To avoid this problem, always use buffered solutions and monitor possible pH modifications introduced by the established protocol, in preliminary experiments. For a more accurate estimate of evoked vesicle release, when analyzing data, monitor the fluorescence signal in a region of the cell surface without fusion events, and use modifications of the fluorescent signal within this region as adjustment factor.

In conclusion, a method for monitoring and analyzing vesicle fusion and dynamics has been described. This technique can be used in different cell types (neurons and endocrine cells) to visualize and dissect the various steps of exo/endocytosis, to reveal the role of proteins and their pathogenic mutants in the regulation of vesicle dynamics and to uncover the mechanisms of action of drugs targeting constitutive and regulated exocytosis.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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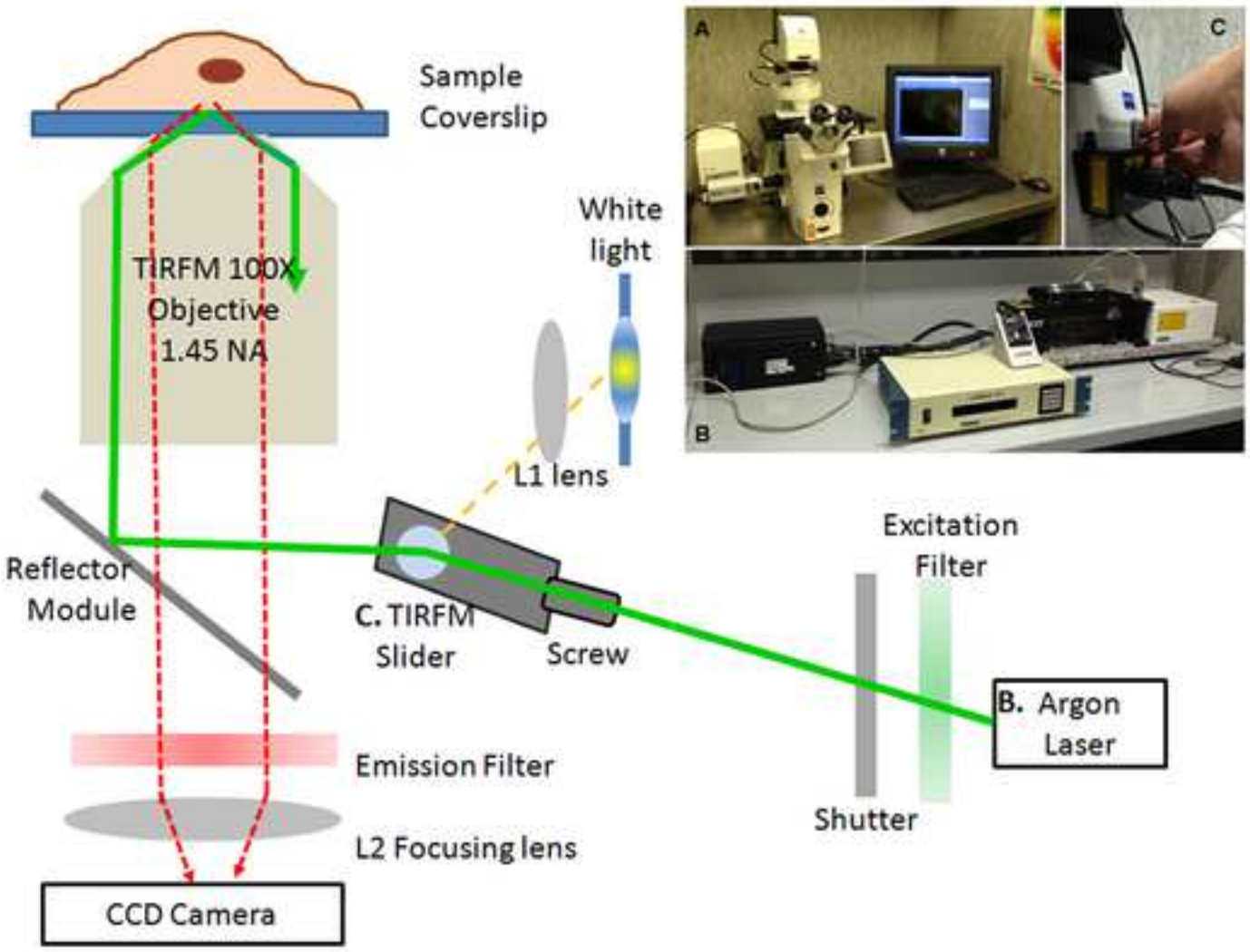

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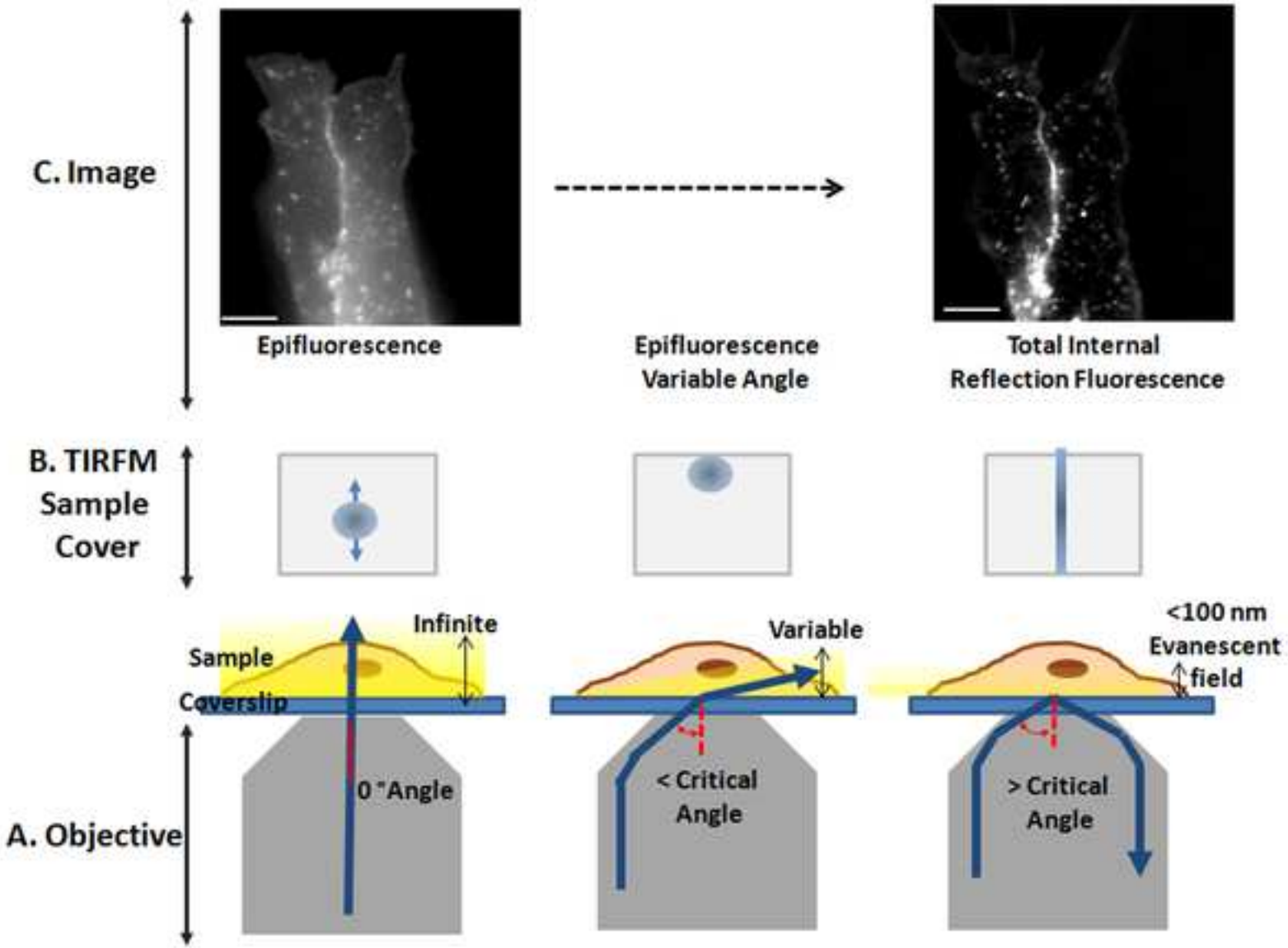

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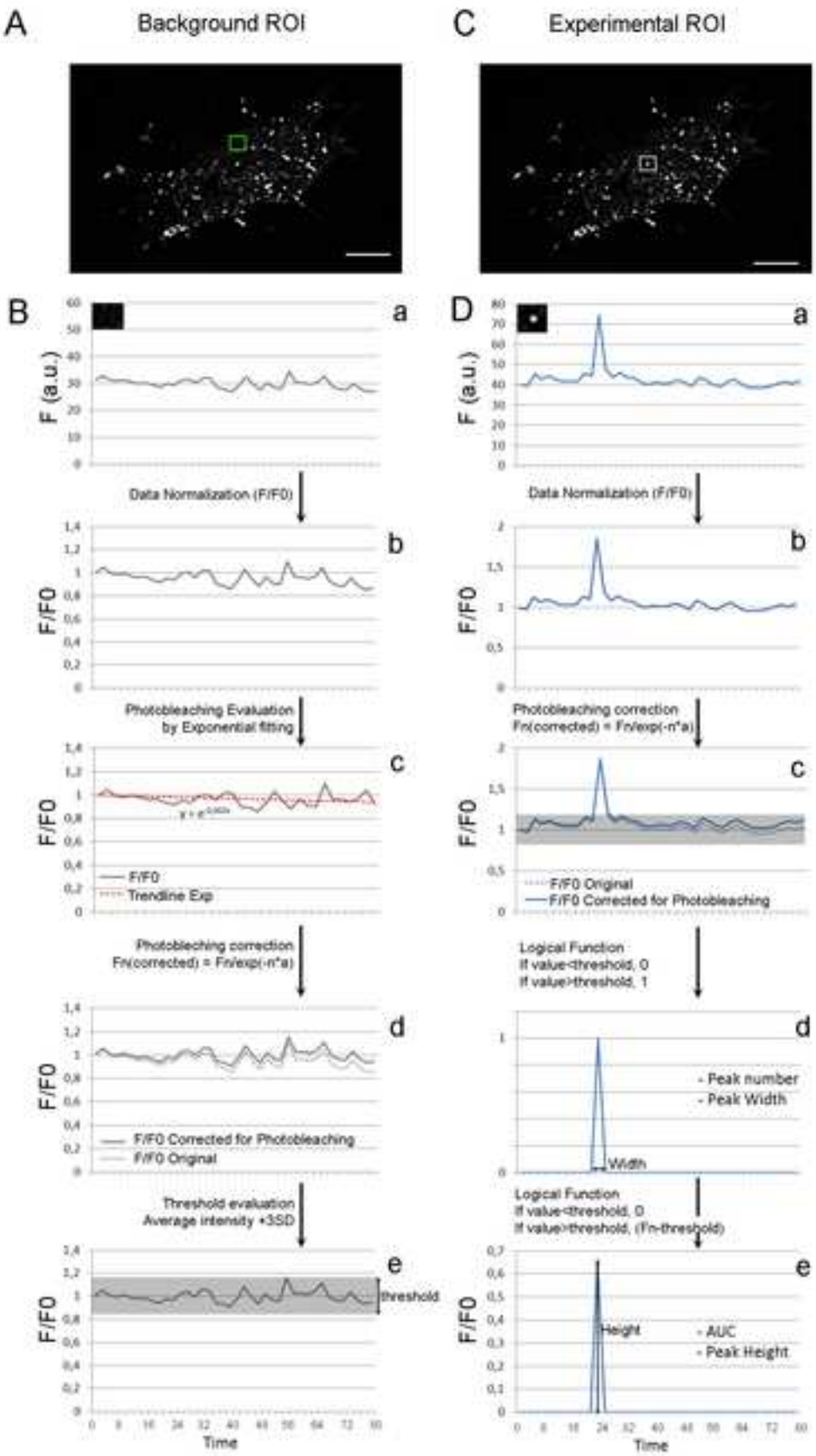

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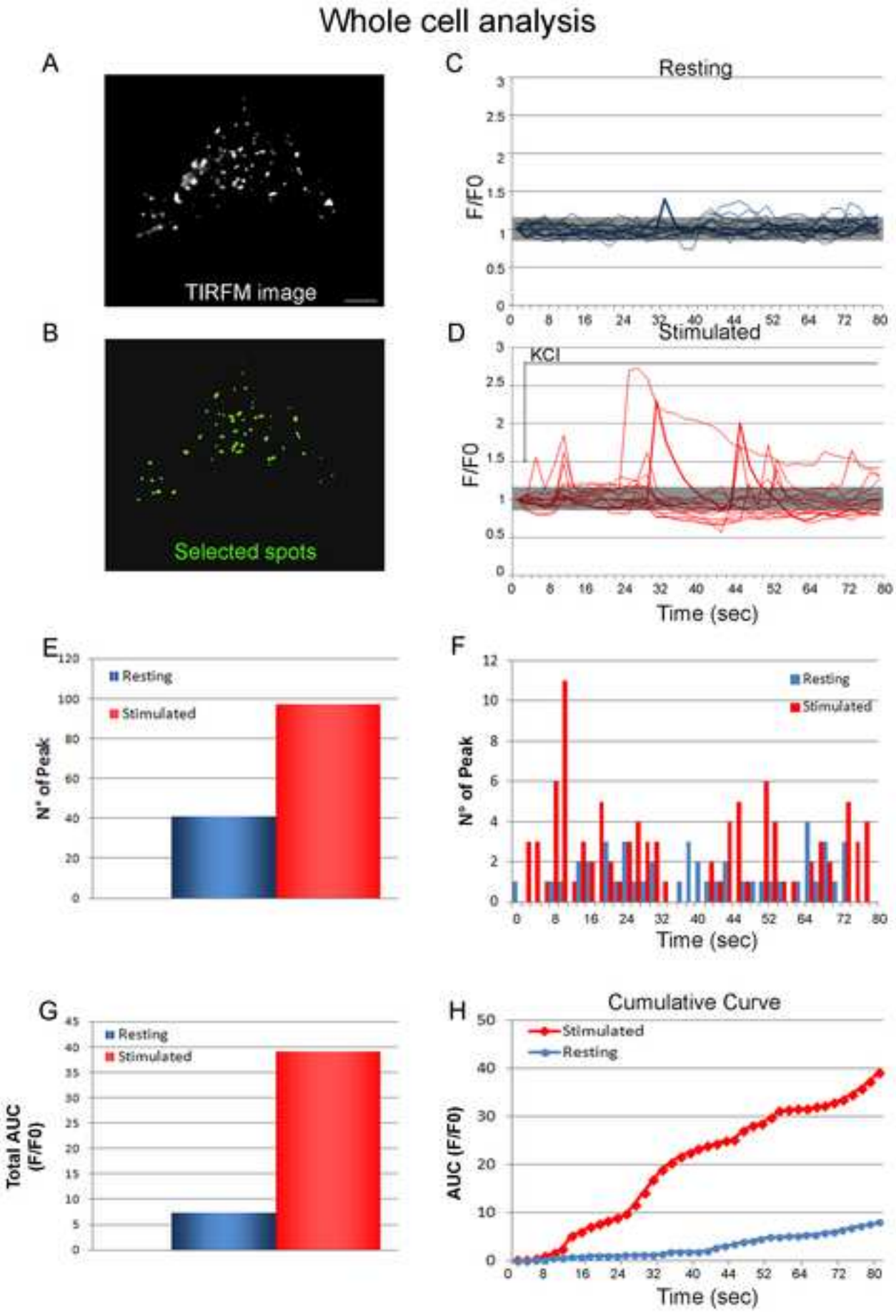

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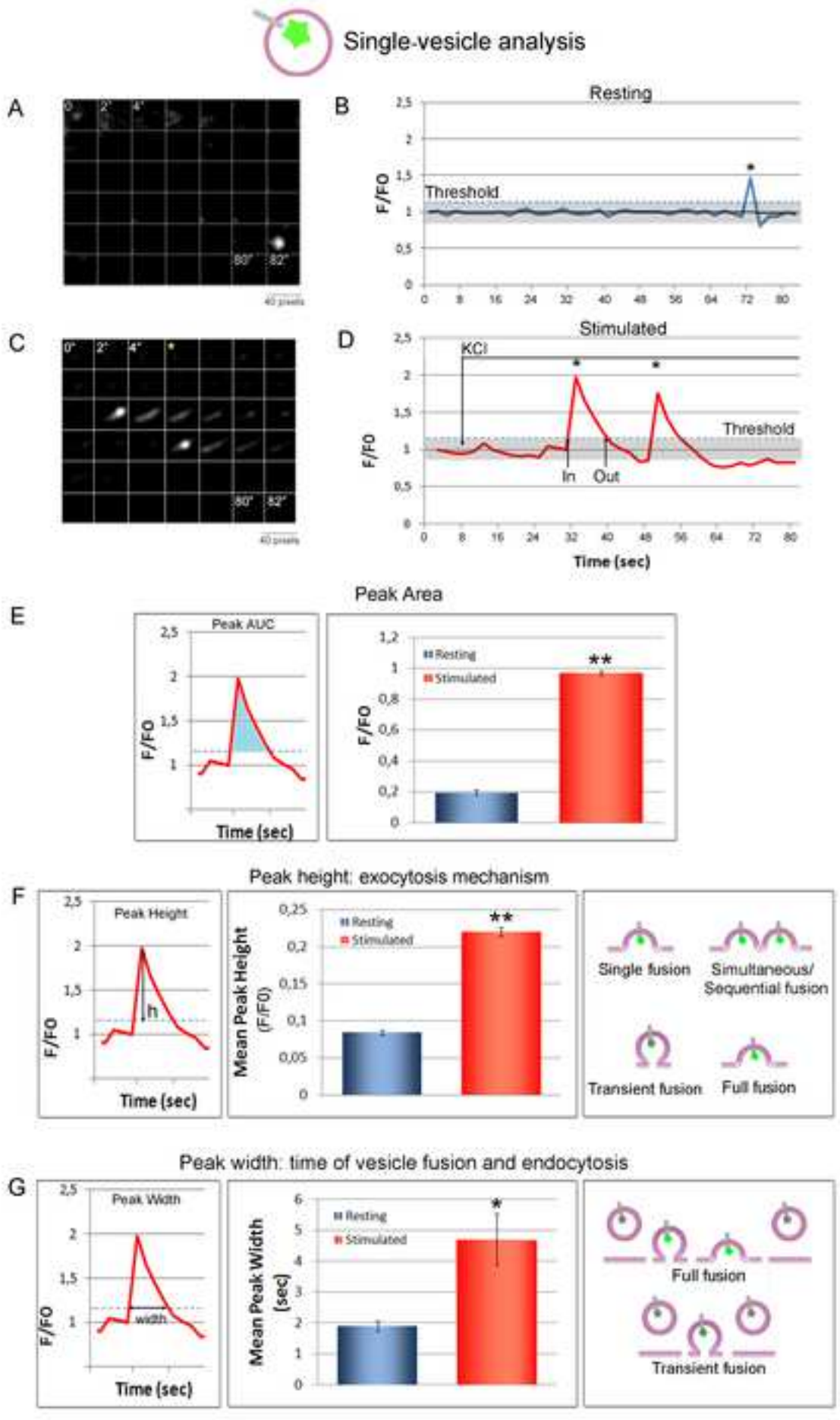

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
Reagent		
SH-SY5Y	ATCC	CRL2266
DMEM HIGH Glucose	Euroclone	ECB7501L
FBS	Euroclone	ECS0120D
Penicillin/streptomicyn	Euroclone	ECB3001D
trypsin 0,05% EDTA 0,02%	Euroclone	ECM0920D
Glutamine	Euroclone	ECM0095L
Na Pyruvate	Sigma-Aldrich	S8636
PEI (polyethylenimine)	PolySciences, Inc	23966
NaCl	Sigma-Aldrich	S5886
KCl	Sigma-Aldrich	P5405
CaCl2	Sigma-Aldrich	C5670
Hepes	Sigma-Aldrich	H4034
MgSO4,	Sigma-Aldrich	M2643
KH2PO4	Sigma-Aldrich	P5655
Glucose	Sigma-Aldrich	G0350500
PureLink HiPure Plasmid Miniprep Kit	Invitrogen- Life Technologies	K2100-03
Disposable		
glass coverslips	Thermo Fisher Scientific	10015871
3.5 cm Petri dish	Euroclone	ET2035
75 cc flask	Sarstedt	83.1811
Equipment		
Axio Observer Z1	Zeiss	491912-9850-000
Multiline Argon Laser Lasos 77	Lasos	00000-1312-752
Laser TIRF slider	Zeiss	423681-9901-000
100x Objective	Zeiss	421190-9900-000
CCD Camera RetigaSRV Fast 1394	QImaging	
LAMBDA 10-3 optical filter changer with SmartShutter	Sutter Instrument Company	

Software

Image ProPlus 6.3 Software	Media Cybernetics
Excel	Microsoft
GraphPad Prism 4.00	GraphPad Software, Inc.

Comments/Description	URL
heat inactivated	http://www.lgcstandards-atcc.org/products/all/CRL-2266.aspx?geo_country=it http://www.euroclonegroup.it/Cell_Biology/Cell_Culture/prodotti/3+2285/Standar http://www.euroclonegroup.it/Cell_Biology/Cell_Culture/Sera/prodotti/4+2283/Fo http://www.euroclonegroup.it/Cell_Biology/Cell_Culture/Reagents_Supplements/p http://www.euroclonegroup.it/Cell_Biology/Cell_Culture/categorie/4+1749/Reager http://www.euroclonegroup.it/Cell_Biology/Cell_Culture/Reagents_Supplements/p http://www.sigmaaldrich.com/catalog/search?interface=All&term=S8636&N=0&m http://www.polysciences.com/Catalog/Department/Product/98/categoryid--505/pr http://www.sigmaaldrich.com/catalog/product/sigma/s5886?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/sigma/p5405?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/sigma/c5670?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/sigma/h4034?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/sigma/m2643?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/sigma/p5655?lang=it&region=IT http://www.sigmaaldrich.com/catalog/product/fluka/g0350500?lang=it&region=IT http://www.lifetechnologies.com/order/catalog/product/K210003?ICID=search-k21
size 22 mm diameters; thickness D=0.17 mm +/- 0.005 mm	http://webshop.fishersci.com/insight2_it/getProduct.do?productCode=10015871 http://www.euroclonegroup.it/Disposable_Material/Plasticlabware/Cell_Culture/pr http://www.sarstedt.com/katalog/en/index.html#/106/
inverted microscope multi-line (458/488/514 nm), 100mW argon-ion laser Oil, NA 1.45 Alpha-Plan	http://www.zeiss.com/microscopy/en_de/products/light-microscopes/axio-observe http://www.lasos.com/products/argon-ion-laser http://www.zeiss.com/microscopy/en_de/products/imaging-systems/single-molecu https://www.micro-shop.zeiss.com/?l=en&p=us&f=o&a=v&m=a&id=421190-9900-(http://www.qimaging.com/products/datasheets/Retiga-SRV.pdf http://www.sutter.com/IMAGING/lambda103.html

spot selection, ROI selection, fluorescence intensity determinatior	http://www.mediacy.com/index.aspx?page=IPP
photobleaching correction, whole-cell and single-vesicle analyses	http://office.microsoft.com/it-it/excel/
statistical analysis	http://www.graphpad.com/scientific-software/prism/

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rodotti/4+2279/Antibiotics.html
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rodotti/4+2278/Aminoacids_Vitamins.html
ode=match%20partialmax&focus=product&lang=it&region=IT
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CORRESPONDING AUTHOR:

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Article Title:	Total internal reflection fluorescence microscopy and pH-sensitive GFP-based sensors to evaluate neurotransmitter vesicle dynamics
Signature:	Carla Perego
Date:	April 25th, 2014

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UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE FARMACOLOGICHE
E BIOMOLECOLARI - DiSFeB

Milan, August 10, 2014

To Jove,
Object: Rebuttal Letter,

We carefully revised our manuscript entitled "**TIRFM and pH-sensitive GFP-based probes to evaluate neurotransmitter vesicle dynamics in the SH-SY5Y neuroblastoma cell line: cell imaging and data analysis**",
Changes in the text are tracked in red.

Major modifications:
- Abstract has been modified
-Protocol and results sections: comments reported in the figure legend in the past version, have been moved to protocol or result sections, according to reviewer suggestions.
- Discussion. A part relative to modification of cell morphology by temperature has been introduced.
The paragraph relative to cellular model has been moved in the first part of discussion

Point-to-point response to criticisms:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The text has been checked for spelling or grammar issues.

2. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, *i.e.,* the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
There are currently 3.75 pages highlighted of Protocol text.
The highlighted text has been reduced to 2.75 pages

3. Please revise the text to avoid the use of any personal pronouns (*e.g.,* "we", "you", "our" *etc.*).
We have deleted all personal pronouns through the text.

4. Please provide units for all numbers throughout the manuscript:
There is only a number without units
Line 473: mean peak height 0.042 ± 0.005 normalized fluorescence intensity
Normalized immunofluorescence intensity has been now inserted in the text (thanks).
It was erroneously deleted using our revision.

5. For example: "a statistically significant increase in the average peak area, peak height and width were detected under stimulated conditions (3.8 ± 0.2; 2.75 ± 0.03 and 2.6 ± 0.5 fold increase over resting conditions, respectively)"
Fold increase is the ratio between two numbers with identical units, by definition is a pure number.
The P value has been inserted.

6. The Figure Legends should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
Details in the figure legend have been moved to results or method sections.

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7. Please move all URL's to the Table of Materials and Equipment.
The URL's have been moved to the Table of Materials and Equipments.

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August 8th, 2014
Object: Rebuttal Letter,

Please find enclosed a point-to-point answer to reviewer's comments.
We would like to thank all reviewers for helpful suggestions.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Authors used combination of pH sensitive DNA encoded sensors and TIRFM imaging technique to explore vesicle recycling events in vivo. Authors recorded imaging in two conditions; vesicle dynamics under resting as well as stimulated conditions. Authors found that as expected more florescence intensity in stimulated condition compared to resting suggesting that more vesicles fused with the plasma membrane. Authors did a great job by using similar cells for resting and stimulated conditions. Authors also described methods and results sections well. However, I would like to mention some comments here.

Major Concerns:
1. I do not think authors developed this technique and it was published before. I would like to ask authors what is the new concept here in this paper. Authors should explain what is the difference between their method compared to previous published method. The modifications should be reported in this paper.

Editor's Note: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.
Author's Note: the TIRFM approach is not novel but few works directly address the mechanisms of vesicle fusion and recycling with this technique in primary neuronal cultures. This is because TIRFM requires cells which grow adherent to a glass cover and are sufficiently flat to allow stable visualization of membranes and fusion events.
We demonstrate for the first time that the SH-SY5Y human neuroblastoma cell line can be a valuable model to investigate neurotransmitter release under resting and stimulated conditions, with the TIRFM approach.
We provide a protocol for growing these cells adherent to the glass cover, which is paramount for TIRFM.
Finally, we describe a semiautomatic procedure, developed in the laboratory, for image processing and data analysis, at whole-cell and single-event levels.
The efficacy of the protocols is highlighted by results presented which demonstrate that potassium depolarization induces a massive release of neurotransmitter (known) and changes the behavior/kinetics of fusion events (new). The stimulus-dependent modification of fusion mechanisms is an emerging idea and has been recently found also in other secretory cell types by TIRFM (synaptic-like microvesicles exocytosis in endocrine cells of the pancreas. Bergeron t al, 2014)

2. Did authors use any drugs which inhibit or delay exocytosis events and see the fluorescence intensity decreases with inhibitors as shown KCl increased fluorescence intensity.
The imaging approach and data analysis were developed in our lab in order to verify the impact of newly identified vesicle proteins and their pathological mutants on neurotransmitter exocytosis.
Using different constructs and the described methods, we were able to measure both an increase and a decrease of basal synaptic activity.

Minor Concerns:
1. authors need to report in the long abstract what they found. Needs to mention results in one or 2 sentences in abstract.
Following reviewer suggestions, the abstract has been modified. Now a consistent part of the abstract is devoted to the developed methods and to results.

2. Although authors explained trouble shooting about morphological changes and fluorophore, authors did not discuss about limitations of method.
A paragraph related to limitations of method has been inserted in the introduction (data analysis) and in the discussion sessions (accurate selection of the cellular model, given that TIRFM can work only with firmly attached cells).

Reviewer #2:
The manuscript by Federica et al., provides a rather detailed description of the technique using total internal reflection fluorescence microscopy (TIRFM) and fluorescent protein expression for visualization and quantification of vesicle release in adherent secretory cultured cells. TIRFM and its applications to study vesicle release are not novel and have been amply described in the literature before. However, this paper/video will still benefit a potential novice user if it includes a more thorough yet generalized description of the protocol and likely pitfalls to be encountered.

Major comments:
1. The protocol cannot be easily followed. It has too many details describing the TIRF setup, while the algorithms for data analysis are not well presented.
The TIRF setup has been reduced to essential information.
The algorithms for data analysis have been developed, a workflow for image processing and data analysis has now been included in figure 3 (New figure).

a. In the protocol, the section 1.1 is best to be split into two sub-sections, one concerting SH-SY5Y culture maintenance and the other cell coverslip plaiting. Also, please specify the thickness, glass type and diameter of coverlips optimal for TIRFM, any special coverslip pre-treatment (poly-d-lysine, collagen, etc. or none). In all protocol sections, please provide specific estimated volumes of solutions one might need per experiment or a plate of cells instead of saying "opportune volume". Do you maintain cells on glass coverlips or on plastic please make it clear? Do you filter or otherwise sterilize your solutions?

- Section 1.1 has been splitted in two sections: “SH-SY5Y cell culture” and “cell plating for imaging analysis”
- Coverslips characteristics are specified in the text and in the “Table of Materials and Equipment”
- Coverslip pre-treatment has now been specified in the method, no coating material is used.
- All solution volumes are specified through the text.
- The requested details about cell maintenance and sterilization of solution have been added.

b. Section 2 needs to include only essential details about the TIRFM system necessary to interpret the results presented in the manuscript , but still sufficient to replicate the findings in a different TIRF system (illumination source, excitation/emission filters, dichroic mirror, objective lens specs, CCD camera. The sample preparation (2.2) and sample imaging (2.3) sections can be merged and shortened to just essential steps. When is typically pHluorin expression sufficient for imaging?

- Section 2, now includes only essential information about the microscope setup
- Sample preparation and imaging have been merged and shortened.
- A sentence about optimal pHluorin expression for imaging has been added (paragraph 2.2.5)

c. In section 3, what program is used for image analysis, ImageJ? Where does one download it from with the right set of plug-ins? Provide a brief description of the code/algorithm used in the macro. Define roundness score or cite an article where it can be found.

Editor’s Note: Please provide download URL's in the Table of Materials and Equipment.
Author’s comment:
The code/algorithm has been described.
We are not allowed to use commercial language through the text (journal policy), but a list of softwares employed and corresponding URL’s is reported in the “Table of Materials and Equipment”.
The following programs have been used:

-Image Pro-Plus, Media Cybernetics software for image analysis (spot selection, ROIs selection, fluorescence intensity determination).
- Excell, Microsoft for photobleaching estimation and correction, whole-cell and single-vesicle analyses. The macros have been developed in our lab using logical and mathematical functions already defined in the Excell program.
- Excell/Prism3 for statistical evaluation.

The specific macros developed in the lab are available on request.
A list of equivalent macros, freely available, is reported at the URL:
http://rsbweb.nih.gov/ij/docs/guide/index.html (Table of Materials and Equipment).

2. It is known that strong stimuli lead to a calcium-dependent acidification of the cytosol and mirrored alkalization in the extracellular space via PMCA see Rossano et al., 2013. It is thus likely that extracellular pH increases during KCl stimulation, especially in a tiny volume between the cell and the surface of the coverlslip leading to the exaggeration of pHluorin fluorescent signal and the calculated values of evoked vesicle release. This issue needs to be addressed in the discussion. For a more accurate estimate of evoked vesicle release, one would also need to perform measurements of plasma membrane expressed pHluorin fluorescent responses to a given KCl concentration, and use it as an adjustment factor.

This part has been addressed in the discussion (fluorophores lines 643-648).
We always place a “background” ROI on a surface region of the cells without synaptic events. This helps us in monitoring and estimate possible fluorescence variations induced by stimulation.

Minor fixes:

Line 57: fluorescent pH-sensitive DNA-encoded sensors
Line 59: with synaptobrevin is expressed in cultured cells to label the lumen of synaptic vesicles
Line 101: to proteins in the vesicle membrane
Line 102: include a sentence on the kinetics of pHluorin responses (faster than a fusion event)
Line 126: TIRFM
Line 128: "The simultaneous recording of pixels" replace with "Chip-based image acquisition"
Line 166: Fix EDTA spelling
Line 203: Petri dish containing coverslips with cells
Line 240: KCl-KRH provide exact concentrations for every component
Line 358/384: "peak width" not "peak wide"
Line 377: "Fluorescent signal from a single fusion event is shown"
Line 389: as during the control recording
Line 398: width not spread?
Line 403: cumulative fluorescence values
Line 422/433: show a square in the image not a circle
Line 469: define "TIRF zone"
Line 476: the evanescent field with the depth of 70-200 nm
Lines 494-505: need to be merged and rewritten more clearly

All minor concerns have been fixed. Thanks.

Line 424/436: You imaged control and KCl experiments under the same conditions - 1Hz acquisition rate. In Fig 3B (70-80 frame) and Fig 4B show only relevant frames (do not show every frame).

The acquisition rate was 1Hz, but only every second frame was shown in the figure 4B (now reported in figure 5A and 5C). A sentence has been added to explain this discrepancy.

Line 514: Please mention that bleaching in many instances follows a simple exponential decay function, which makes it easier to estimate it and correct for it by performing control recordings.

A sentence explaining that bleaching in many instances follows a simple exponential decay function has been added in the discussion (bleaching paragraph).

Reviewer #3:

Manuscript Summary:

The manuscript attempts a step-by step description of procedures that involve a combination of biophysical and cell biology techniques and allow registration of synaptic vesicles exocytosis using an advanced imaging approach TIRFM in cultured neuroblastoma cells transfected with a genetically encoded optical indicator of vesicle release and recycling pHluorin, as well as data analysis focused on assessment of these processes' dynamics.

Major Concerns:

The clarity of presentation is the most problematic part of the submitted manuscript.

Its illustrations do not present sufficient details and explanations that would allow another researcher in the field to easily comprehend and replicate procedures.

As suggested, the illustrations have been modified.

- Figure 1 has been modified, now it includes a schematic view of the TIRF microscope set-up. Our system is shown in the inset.
- Figure 2 has been modified. Now it describes the procedure to achieve TIRF illumination.
- Figure 3. A new figure illustrating the workflow of image processing and analysis has been added.
- Figure 4 has been modified. Now it describes whole-cell analysis in resting and stimulated cells
- Figure 5 has been modified. Now it describes single-vesicle analysis in resting and stimulated cells

Procedure called "2.1) Perform TIRF imaging with a commercial TIRF system" (line 211), and illustrated with a still picture of their microscope, does not quite meet expectations/definition of a single step of multistep description of the process.

The figure 1 has been modified, now it includes a schematic view of the TIRF microscope set-up. Our system is shown in the inset.

Yet, I believe that both technical quality and efficiency of the presentation could be improved by the authors to the extent that would make this article a useful source for other researchers.

Minor Concerns:

It would help for the clarity of the report to having a native English speaker proof-reading the article before submission.

The beginning part of the Abstract(s), as well as a large portion of the Introduction, is devoted to molecular mechanisms of synaptic transmission, leaving less space for introducing the described techniques.

The first part of abstract and introduction have been modified

Please check terminology used in the paper. E.g., voltage sensitive Ca2+-channels (line 73), isn't an established term. You may use either voltage-gated or voltage-dependent instead.

This part has been eliminated. The terminology in the paper has been checked.

Reference "9" (lines 101, 108 and 186) does not describe pHluorin-s or discuss their application to study vesicle recycling, but rather introduces TIRF microscopy.

By mistake, in the old version, the reference list was shifted. Now the reference 9 refers to the paper of Miesenböck and coworkers (Nature. 394(6689):192-5, 1998) which describes pHluorin-s and discusses their application to study vesicle recycling.

SH-SY5Y cell culture section does not refer to any previously described procedures, and provides no comment about its possible modification by the authors.

The paper reporting the original description of SH-SY5Y culturing has been included in the text and in the reference list. Modifications introduced by our laboratory have been reported in the protocol.

Instead of using one section describing configuration of the microscope and all settings used for imaging, authors list them as four procedural steps with sentences starting with the same " Use… wording (line 211, 215, 219 and 224).
The section “microscope set up” has been modified and reduced to the essential. It is still listed as more procedural steps because as requested by the Science editor.

Section 2.2) is called "Sample preparation", but it actually talks about "Turning lasers on" (line 230) or "performing imaging" (line 243).
Section 2.2 has been modified, it contains the procedure for getting TIRF illumination and is called “How to achieve TIRF illumination”

This section also suggests "2.2.5) to stabilize the imaging chamber" (line 248), but does not explain how or what does it mean/involve, as well as utilize an obscure term/word "opportune" (line 234).
After having reached TIRF conditions, leaving the object in contact with the coverslip few minutes before starting TIRFM imaging, reduces the presence of interference fringes, in our experience.
All terms “opportune” in the text have been substitute with the exact volume of solution.

Line 255 (2.3.1) does not specify meaning of switching to "in live mode". Does it refer to hardware or software setting?
It refers to a software setting. The term has been specified in the text

Line 263 (2.3.2) suggests using "established imaging frequency" without explaining or referring to procedures that establish it.
The imaging frequency has been specified.

Line 280 (3.1.1) would benefit from mentioning software that runs the referred macro.
We are not allowed to use commercial language through the text (journal Policy). The softwares used have been specified in “Table of Material and equipment”.
-Image Pro-Plus, Media Cybernetics software for image analysis (spot selection, area selection, fluorescence intensity determination).
- Excell,Microsoft for photobleaching correction, whole-cell and single-vesicle analyses. The macros have been developed in our lab using logical and mathematical functions already defined in the Excell program.
- Excell/Prism3 for statistical evaluation.
The URL’s of downloadable macros has been added (3 section)

Additional Comments to Authors:
Provided 2 videos do not demonstrate any particular procedures described in the paper, but rather show examples of their recorded data.
It would be of advantage for authors to look at a couple other JoVE papers with videos before submitting their new revised version.
The video showing the procedures described in the paper will be recorded by JoVe, once the paper will be accepted.
With the provided videos, we just meant to give a general demonstration of our data and analysis.
However, following your suggestions, we illustrate the procedures in the new figures.

Listed above drawbacks are just examples of many other instances of confusing description that misses details, references or connections to other parts of the paper.
We did our best to try to fix all drawbacks present in the paper.