Video Article

Lateral Diffusion and Exocytosis of Membrane Proteins in Cultured Neurons Assessed using Fluorescence Recovery and Fluorescence-loss Photobleaching

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Abstract

Membrane proteins such as receptors and ion channels undergo active trafficking in neurons, which are highly polarised and morphologically complex. This directed trafficking is of fundamental importance to deliver, maintain or remove synaptic proteins.

Super-ecliptic pHluorin (SEP) is a pH-sensitive derivative of eGFP that has been extensively used for live cell imaging of plasma membrane proteins[1-2]. At low pH, protonation of SEP decreases photon absorption and eliminates fluorescence emission. As most intracellular trafficking events occur in compartments with low pH, where SEP fluorescence is eclipsed, the fluorescence signal from SEP-tagged proteins is predominantly from the plasma membrane where the SEP is exposed to a neutral pH extracellular environment. When illuminated at high intensity SEP, like every fluorescent dye, is irreversibly photodamaged (photobleached)[3-5]. Importantly, because low pH quenches photon absorption, only surface expressed SEP can be photobleached whereas intracellular SEP is unaffected by the high intensity illumination[6-10].

FRAP (fluorescence recovery after photobleaching) of SEP-tagged proteins is a convenient and powerful technique for assessing protein dynamics at the plasma membrane. When fluorescently tagged proteins are photobleached in a region of interest (ROI) the recovery in fluorescence occurs due to the movement of unbleached SEP-tagged proteins into the bleached region. This can occur via lateral diffusion and/or from exocytosis of non-photobleached receptors supplied either by *de novo* synthesis or recycling (see Fig. 1). The fraction of immobile and mobile protein can be determined and the mobility and kinetics of the diffusible fraction can be interrogated under basal and stimulated conditions such as agonist application or neuronal activation stimuli such as NMDA or KCl application[8,10].

We describe photobleaching techniques designed to selectively visualize the recovery of fluorescence attributable to exocytosis. Briefly, an ROI is photobleached once as with standard FRAP protocols, followed, after a brief recovery, by repetitive bleaching of the flanking regions. This 'FRAP-FLIP' protocol, developed in our lab, has been used to characterize AMPA receptor trafficking at dendritic spines[10], and is applicable to a wide range of trafficking studies to evaluate the intracellular trafficking and exocytosis.

Video Link

The video component of this article can be found at http://www.jove.com/video/3747/

Protocol

1. Cell Culture, Viral Transduction, and Protein Expression

1. Culture high density hippocampal neurons from embryonic day 18 (E18) rat pups on poly-l-lysine-coated glass coverslips for 14-25 days *in vitro* (DIV).
2. 6-24hrs prior to the live experiments, transduce cells with attenuated Sindbis virus containing the membrane protein of interest, tagged with the super-ecliptic pHluorin (SEP).
3. Add the pseudovirion-containing medium directly to the coverslip containing 1mL of conditioned media and returned to the culture incubator. The titer and time for protein expression following viral transduction will vary depending on the virus batch and should be determined for each batch prior to commencing live cell experiments.

2. FRAP-FLIP Live Cell Imaging

1. *Equipment set-up*

1. Transfer the coverslip to the imaging chamber of a Zeiss Axiovert LSM 510 META confocal microscope. To minimize power fluctuations during imaging, ensure the microscope has been switched on, with 100% laser output, for at least 20 mins prior to imaging.
2. Immediately, replace the culture medium with pre-warmed (37 °C) extracellular recording solution containing 140 mM NaCl, 5 mM KCl, 15 mM glucose ,1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20-25 mM HEPES (pH adjusted to 7.4 with NaOH) and place the chamber on the pre-heated stage (37 °C) of the Zeiss Axiovert.
   Ensure the osmolarity of the extracellular recording solution is adjusted to within 10 mOsM of your culture medium. Provided no significant evaporation occurs during the timecourse of the experiment, this CO_2 independent solution is suitable for short experiments (<10 hrs). Supplementing with 1-2 mM sodium bicarbonate is recommended.

2. *Defining the imaging capture parameters*
   1. First, identify a neuron expressing the recombinant protein of interest and bring it into focus.
   2. With a 63X oil objected, acquire an image of the whole cell using 488nm laser light excitation at low laser power. To minimize photobleaching, use a fast nominal speed (7-9) and low pixel resolution (512-512) keeping total scan speed <1 second.
   3. Select a portion of dendrite to image and zoom to capture a frame containing the ROI (~1.5-2.5 x optical zoom). Where possible, ensure the field of view contains several processes so that measurements from reference dendrites can be obtained, to determine if non-specific photobleaching due to acquisition is occurring.
   4. Adjust the filters, pinhole, scan speed and detector gain to enable maximal fluorescence from minimal laser excitation but with limited saturation. A large pinhole diameter is recommended, to maximize photon collection (2µm is suitable for spines and ternary dendrites). The detector gain should be strong enough to detect small fluorescence increments, such that the very first images, before the photobleaching do not overcome 10% of saturated pixels.
   5. Save this configuration to be used for the pre-/ post-bleach and recovery phases of the experiment.
   6. Next define the photobleach regions; selecting an ROI for the initial photobleach and flanking regions for the subsequent repetitive photobleaching phase. Ensure the flanking regions are wide enough to prevent recovery by diffusion between scans (typically 5 µm, see Fig. 2).
   7. Adjust the bleach parameters for both photobleach ROIs. The initial photobleach should be rapid (0.1-0.5 sec) requiring between 1-5 iterations, depending on the optical zoom and the volume of the ROI. For the flanking regions, the laser excitation should be adjusted to ensure continuous photobleaching of the flanking regions, but without phototoxic damage.
   8. As a guideline, we use 100% laser excitation for the initial photobleach, and 10% for the repetitive photobleaching phase.

3. *Image acquisition*
   1. Once all parameters have been set, perform the FRAP-FLIP experiment as a variable time-lapse image sequence, outlined in the 4 blocks below:
      BLOCK 1: 3-10 pre-bleach baseline images, at low laser power, no time delay
      BLOCK 2: Photobleach the central ROI at full laser power, 1-5 iterations
      BLOCK 3: 3-10 post-bleach recovery images
      BLOCK 4: Repetitive photobleach of flanking regions at medium laser power with image capture at a typical time interval of 1 - 5 seconds, depending on the recovery rate of the protein under investigation.
   2. Finally, replace the extracellular recording solution with recording solution buffered at pH6 containing 140 mM NaCl, 5 mM KCl, 15mM glucose ,1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20-25 mM MES (to quench fluorescence) or complemented with 50 mM NH_4Cl containing 90 mM NaCl, 5 mM KCl, 15 mM glucose ,1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20-25 mM HEPES, pH7.4 (to reveal the proteins in low pH intracellular stores), (see Fig. 2).
   3. Collect at least 10 to 20 data sets for each recombinant protein, to enable statistical analysis. To avoid bias results, ensure imaging conditions are maintained consistent across replicates. Discard any data sets where incomplete bleaching, significant focal plane drift or phototoxic cell damage are observed.

3. Data Analysis

1. Open the images with ImageJ software.
2. Align the stacks to account for small fluctuations in the xy plane that may have occurred throughout the time series using the Stackreg plugin (plugins → stackreg →transformation: rigid body → <ok>).
3. For images taken on Zeiss confocal, use the LSM Toolbox plugin to report the time values as a text file (plugins→ LSM Toolbox → Show LSM Toolbox → <apply stamps> → <apply t-stamp>), and import these values into an analysis spreadsheet.
4. To measure the fluorescence fluctuations during the FRAP-FLIP experiment, divide the photobleached segment into individual pixel regions (~20pixels) and measure the mean fluorescence of these ROIs at each time point (F). To quickly obtain these values, select multiple ROIs using the ImageJ 'ROI Manager' analysis tool (Analyze → Tools → ROI Manager) and report the mean fluorescence per pixel using the 'Plot Z-axis profile' command (Image →Stacks →Plot Z-axis profile).
5. Repeat this step to measure the fluorescence intensity of a background, non fluorescent region.
6. Normalize the fluorescence intensity at each time point by subtracting the background values to remove experimental noise, and divide all values by the mean pre-bleached baseline measure.
7. Measure the fluorescence intensity of adjacent non-photobleached dendrites to assess the level of non-specific photobleaching during acquisition. Correction for non-specific photobleaching is discouraged for interpretation of 'FRAP-FLIP' data and should be avoided whenever possible.
8. To calculate if significant recovery has taken place in a ROI, subtract the mean of last 5-10 measures of the sequence from the mean of the first 5-10 measures (ΔF) and determine the statistical significance. Categorize the recovering and non-recovering ROIs to assess the pattern of exocytosis (i.e. exocytosis hotspots or spine vs shaft recovery).

4. Representative Results

The outcome of typical FRAP-FLIP experiment is shown in Fig. 2. Here, a neuron expressing the GluA2 subunit of AMPA type glutamate receptor, tagged with SEP has been selectively photobleached along a region of dendrite. Fig 2.A illustrates the region of dendrite which has been imaged and indicates the ROIs that have been selected for photobleaching (white box regions). The large white boxed area was bleached once, followed by repetitive bleaching of the flanking boxed regions, highlighted with double headed blue arrows. The red arrow indicates the measured ROI, shown in high magnification in the lower panels.

Fig 2.B, shows the fluorescence intensity in the measured ROI over the time course of the experiment. In this example, a one minute recovery period was recorded after the initial photobleach to allow unbleached receptors to enter the ROI by lateral diffusion. When the flanking regions are photobleached, the fluorescence signal from this highly motile fraction of receptors is occluded from the central region, and those within the region are diluted out. The increase in fluorescence (ΔF) observed over the 'FLIP' sequence can therefore be attributed to insertion of SEP-GluA2 in the dendritic shaft. The low pH wash and pH 7.4 + NH4Cl addition respectively confirm that the measured fluorescence is derived from surface proteins and reveal the proportion of intracellular, sequestered proteins within the measured ROI.

In contrast to FRAP, this methodology isolates recovery due to exocytosis, resulting in a greatly reduced level of fluorescence recovery in the photobleached region. To date no reliable mathematical model has been developed to fit and analyze the recovery traces recorded using this selective bleaching technique. It is however, possible to fit the recovery trace with a mono exponential recovery:

$$F_{(t)} = A_s - A_0 e^{(-t/\tau)}$$

Where $F_{(t)}$ is fluorescence at time t, A_s is steady state value, A_0 is the offset at time 0, τ is the time constant. The recovery growth is fixed with a given rate to reach an equilibrium, corresponding to a steady state between insertion and diffusion. Importantly, the time constant extracted from this analysis does not reflect the time constant of exocytosis and can only be used for comparative treatments for an individual protein.

Moreover, the expected pattern of fluorescence recovery is likely to be observed in sub-domains along the photobleached region. Analysis of small ROI within a photobleached segment may be essential to reveal exocytosis "hotspots" and it is advisable to analyse regions of comparable lengths as the variability of density of these spots among the dendrite will affect the calculated rate.

Fig 3 shows an extension of this protocol, applying the photobleach to large section ROI with multiple dendrites in the field of view, followed by selective repetitive bleaching of only one dendrite. This approach enables traditional 'FRAP' and 'FRAP-FLIP' data to be acquired in parallel. In this example, hippocampal neurons have been infected with a glutamate receptor subunit of the kainate class; SEP-tagged GluK2. Prior to imaging, these neurons -were treated with cylcohexamide (2hrs at 200µg/ml), to block protein synthesis. As such, fluorescence recovery in the respective measured ROIs (Fig 3.B) reveals the proportion of recovery due to lateral diffusion and recycling in the standard FRAP dendrite versus recycling alone in the dendrite subjected to the 'FRAP-FLIP' protocol. Comparing the ΔF values from the FRAP versus 'FRAP-FLIP' recovery curves, the relative contributions of recycling (ΔF_{rec} = 11.05%) versus lateral diffusion (ΔF_{diff} = 9.35%) can be inferred. Unlike Figure 2, the recovery does not maintain a steady state level but rather, due to the inhibition of protein synthesis, shows a transient increase and subsequent drop-off in signal, corresponding to depletion of the available receptor pool (approximately 20% decline observed over the recording period).
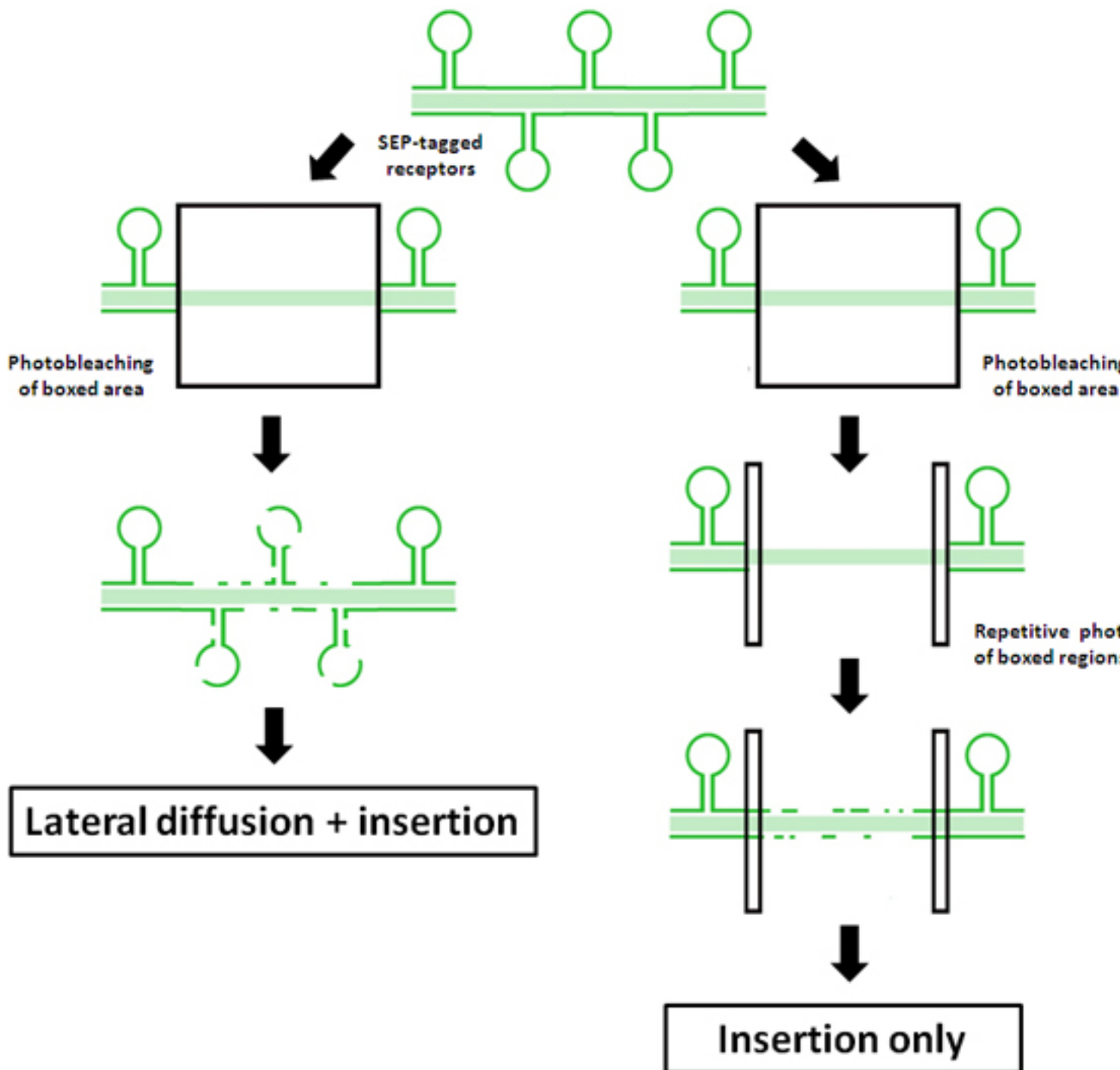
Figure 1. Schematic of the principals of FRAP vs FRAP-FLIP protocols This schematic illustrates the outcomes of a regular FRAP versus a 'FRAP-FLIP' protocol, using SEP-tagged receptors. Fluorescence recovery in traditional FRAP is shown on left-hand side. Measured fluorescence recovery in the central ROI is attributed to a combination of lateral diffusion f non-photobleached SEP-tagged receptors from outside the photobleached ROI and insertion of receptors through recycling and/or *de novo* exocytosis into the dendritic shaft. By contrast, the repetitive photobleached of the flanking ROIs illustrated in right-hand side, shows how this modified 'FRAP-FLIP' protocol silences recovery due to lateral diffusion. As such, any measured fluorescence recovery can be attributed to direct insertion in the ROI.
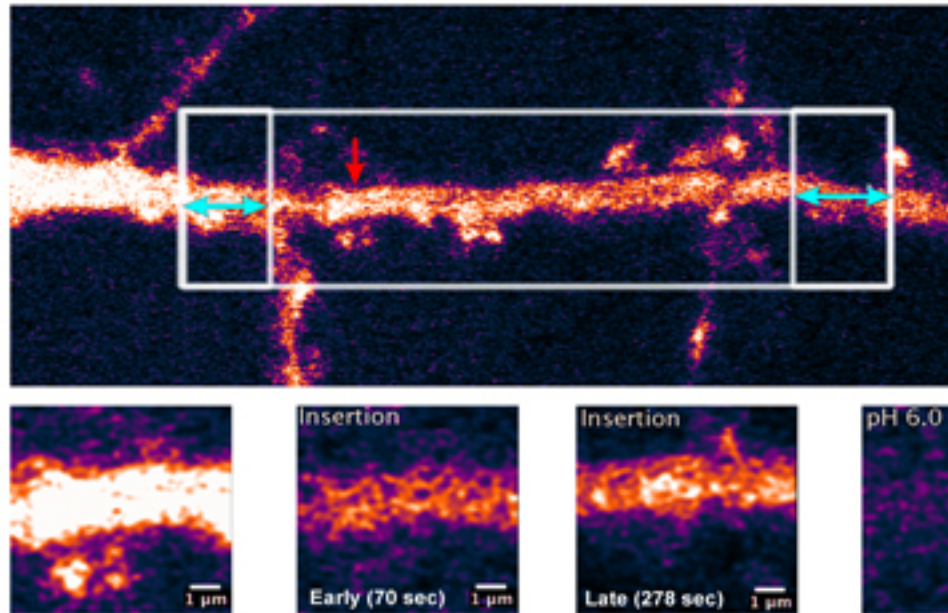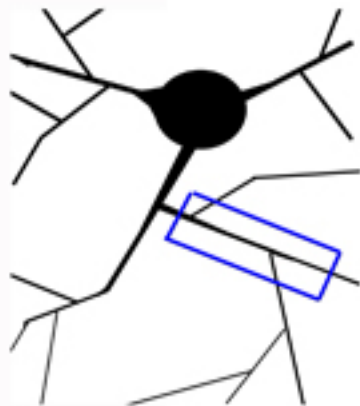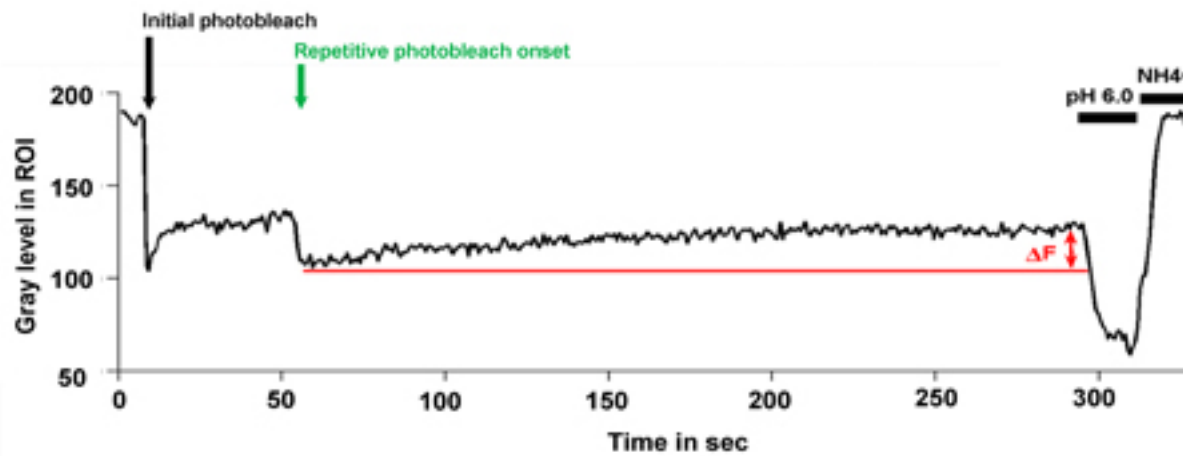
Figure 2. Insertion of SEP-GluA2 into the plasma membrane on the dendritic shaft

A) A hippocampal neuron expressing SEP-GluA2, selectively photobleached along a region of dendrite. The schematic illustrates the region of dendrite which has been imaged, while the upper left hand panel highlights the ROIs that have been selected for photobleaching. The large white boxed area was bleached once, followed by repetitive bleaching of the flanking boxed regions, highlighted with double headed blue arrows. This panel shows the dendrite prior to photobleaching. The red arrow indicates the measured ROI, shown in high magnification in the *lower panels. B)* shows the fluorescence intensity in the measured ROI over the time course of the experiment, plotted as gray level in the ROI (not normalized). The black arrow highlight the timepoint of the initial photobleach and green arrow indicates the start of the repetitive photobleaching. ΔF indicates the increase in fluorescence observed over the recovery period, due to insertion of SEP-GluA2 into the dendritic shaft. Subsequent low pH and pH 7.4 + NH$_4$Cl washes confirm that the recovered fluorescence relates to surface expressed receptors.
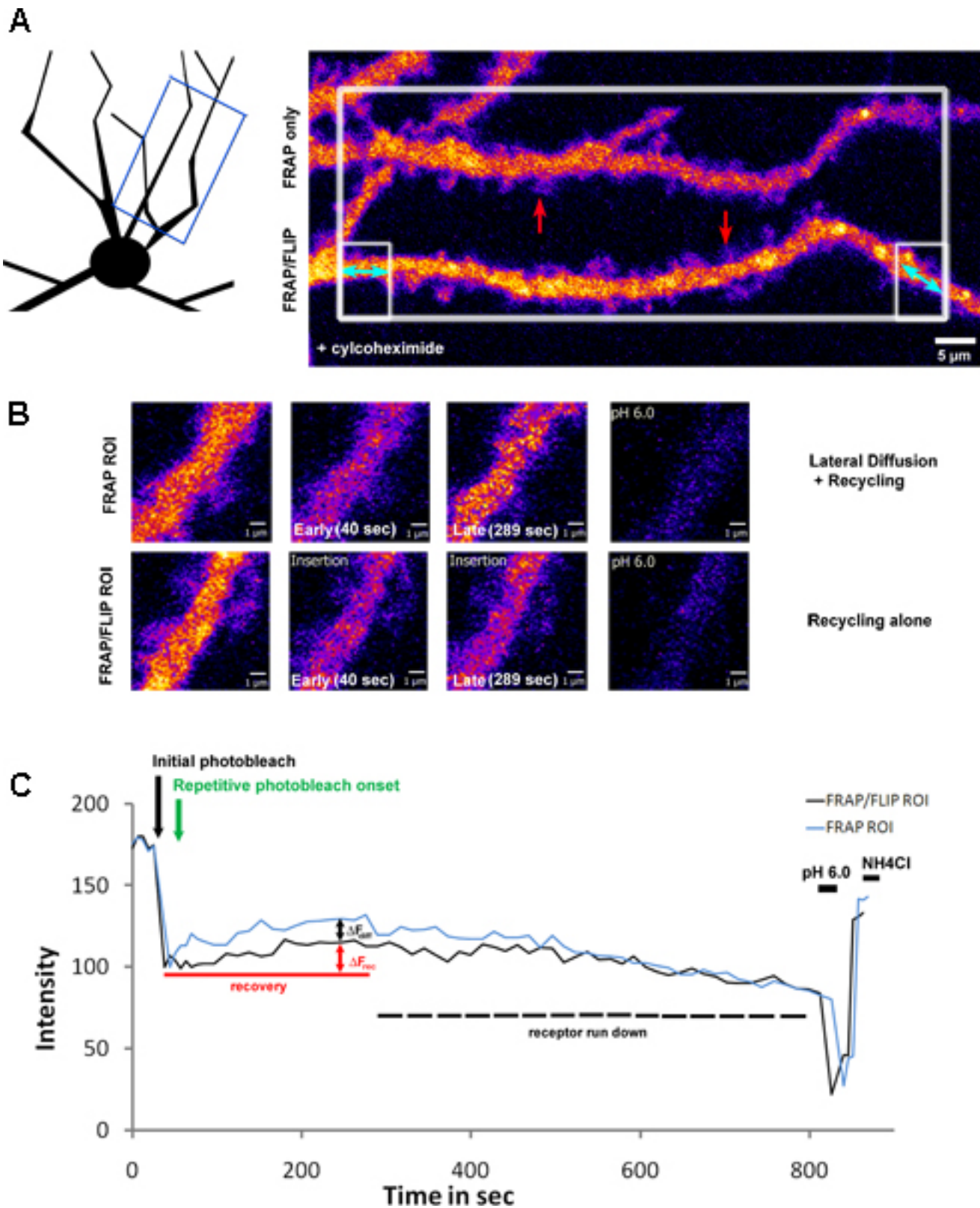
Figure 3. Recycling & lateral diffusion vs Recycling of SEP-GluK2 on the dendritic shaft following cyclohexamide treatment

A) A hippocampal neuron expressing SEP-GluK2, selectively photobleached with parallel FRAP and 'FRAP-FLIP' recovery protocols performed along separate dendritic ROIs. This neuron was subject to pretreatment with cyclohexamide, to block protein synthesis (2 hrs at 200 µg/ml). The schematic illustrates the region of dendrite which has been imaged, while the left hand panel highlighting the ROIs that have been selected for photobleaching. The large white boxed area was bleached once, followed by repetitive bleaching of the flanking boxed regions, of the lower

dendrite only, highlighted with double headed blue arrows. This panel shows the dendrites prior to photobleaching. Red arrows highlight the ROIs shown in high magnification in *B)* illustrating the fluorescence recovery in traditional FRAP versus the 'FRAP-FLIP' protocol. Comparisons of levels of fluorescence intensity in the late stage of recovery (third panels) show the contribution of lateral diffusion and recycling versus recycling alone. *C)* shows the fluorescence intensity in the measured ROIs over the time course of the experiment, plotted as normalized intensity values. The black arrow highlight the timepoint of the initial photobleach and green arrow indicates the start of the repetitive photobleaching. ΔF_{rec} indicates the transient recovery due to receptor recycling, determined from the FRAP/FLIP dendrite while ΔF_{diff} measures the contribution of lateral diffusion of SEP-GluK2 into the dendritic shaft in the 'FRAP only' ROI. The transient increase is followed by gradual decline in signal, corresponding to the run down of available receptors as a result of cyclohexamide treatment. The subsequent low pH and pH 7.4 + NH_4Cl washes confirm that the recovered fluorescence relates to surface expressed receptors.

Discussion

We describe an innovative strategy to visualize the components of plasma membrane protein trafficking. The combinatorial approach of photobleaching techniques with SEP-tagged protein enables selectively plasma membrane insertion events to be assessed. By continually photobleaching the membrane proteins in flanking regions during recovery, the 'FRAP-FLIP' method assesses the contribution of vesicular trafficking to fluorescence recovery. This novel approach allows direct recording of protein membrane insertion, enabling both the number of sub-compartments where recovery is observed and the amplitude of the recovery (ΔF) at the steady state to be determined. Further, comparison of FRAP with and without FLIP allows the proportion of recovery attributable to lateral diffusion to be calculated.

Moreover, in the same experiment, regions of non-photobleached dendrite adjacent to the flanking photobleached regions can be qualitatively assessed during the recovery; fluorescence loss observed in these regions will be due to lateral diffusion of photobleached receptors into these non-photobleached segments of the dendrite.

This selective photobleaching protocol can be utilized to investigate a variety of cellular trafficking processes such as characterizing excocytosis in the plasma membrane in defined subareas (for example, dendrites or spines) or assessing the contribution of lateral diffusion vs insertion by conducting parallel FRAP and 'FRAP-FLIP' protocols along adjacent dendrites (Fig. 3).

Clearly, while specific guidelines have been presented, each lab will need optimize the imaging parameters according to specific specimens and equipments. Importantly, all surface receptors in the ROI need to be photobleached, independent of the z-axis focal plane, but without significant phototoxic damage or non-specific photobleaching. Users should exercise caution when attempting this protocol at the cell soma, as the high percentage of relatively low pH intracellular compartments typically results in high background fluorescence in these regions. Moreover, whilst we have demonstrated how this technique can be applied in varies ways, prior to commencing selective photobleaching experiments on a new SEP-tagged construct, we advise that an initial characterization of the tagged receptors is first conducted, as described by Ashby et al[2].

Overall, this method is a powerful and versatile adaptation of the standard FRAP protocol, enabling insertion events in plasma membrane to be evaluated in near real time.

Disclosures

No conflicts of interest declared.

Acknowledgements

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