

Imaging pHluorin-Based Probes at Hippocampal Synapses

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Summary

Accurate measurement of synaptic vesicle exocytosis and endocytosis is crucial to understanding the molecular basis of synaptic transmission. The fusion of a pH-sensitive green fluorescent protein (pHluorin) to various synaptic vesicle proteins has allowed the study of synaptic vesicle recycling in real time. Two such probes, synaptopHluorin and sypHy, have been imaged at synapses of hippocampal neurons in culture. The combination of these reporters with techniques for molecular interference, such as RNAi allows for the study of molecules involved in synaptic vesicle recycling. Here the authors describe methods for the culture and transfection of hippocampal neurons, imaging of pHluorin-based probes at synapses and analysis of pHluorin signals down to the resolution of individual synaptic vesicles.

Key words: Endocytosis; exocytosis; hippocampal synapses; imaging; neurons; synaptic vesicle; synaptopHluorin; sypHy.

1. Introduction

Neurons transmit information at chemical synapses via release of neurotransmitter from synaptic vesicles (1). The exocytosis and subsequent endocytosis of vesicular membrane and proteins has been intensely studied by neurobiologists for more than three decades (2). One of the most widely used preparations for studying synaptic vesicle cycling is hippocampal neurons in culture. Optical methods for measuring exocytosis and endocytosis of synaptic vesicles have

come to the fore owing to the inaccessibility of small synapses to electrophysiological recording methods (3). Of these optical methods, one of the most direct is the imaging of a pH-sensitive green fluorescent protein (GFP), termed *pHluorin*, developed by Miesenböck and others (4,5). The pHluorin can be targeted to the interior of synaptic vesicles by fusion to proteins such as synaptobrevin (synaptotHuoirin) (4), synaptophysin (syphHy) (6), synaptotagmin (7) or VGLUT1 (8). As illustrated in Fig. 1, the pHluorin is quenched by the low pH inside the vesicle (pH \sim 5.5). Upon stimulation, vesicles fuse with the plasma membrane, protons are lost and the fluorescence of pHluorin increases as the pH moves to 7.4. This is seen as an upward deflection in the syphHy trace recording the fluorescence of syphHy in a small region over a synaptic bouton. The fluorescence declines again following endocytosis and vesicle re-acidification. Re-acidification is fast

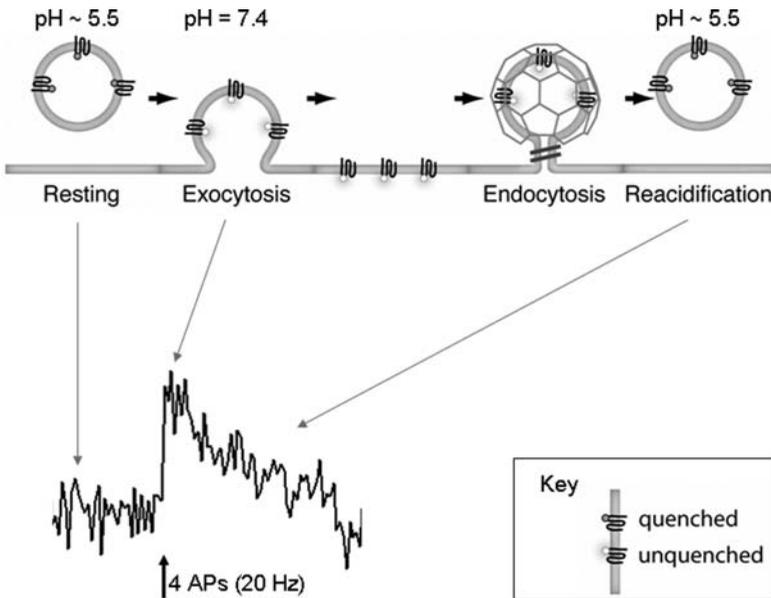


Fig. 1. Schematic of imaging exocytosis and endocytosis with pHluorin-based probes. SyphHy comprises superecliptic pHluorin (pH-sensitive green fluorescent protein) fused to the second intravesicular loop of synaptophysin. The pHluorin is quenched by the low pH inside the vesicle (pH \sim 5.5). Upon stimulation (black arrow), vesicles fuse with the plasma membrane, protons are lost and the fluorescence of syphHy increases as the pH moves to 7.4. This is seen as an upward deflection in the syphHy trace (red). The fluorescence declines again following endocytosis and vesicle re-acidification. Reacidification is fast ($\tau \sim$ 4 s), so the decline in syphHy signals gives us a measure of endocytosis in virtually real time.

($\tau \sim 4$ s), so the decline in sypHy signals gives us a measure of endocytosis that is close to real time (6,9).

The imaging of pHluorin-based probes at hippocampal synapses has been used to describe the kinetics of endocytosis (6,10,11) and to investigate the molecules involved in vesicle retrieval (6). In our hands, sypHy is the best currently available pHluorin-based probe. It is better localized to vesicles and has a greater signal-to-noise ratio compared with synaptopHluorin (6). We have optimized conditions for the culture of healthy hippocampal neurons and for their transfection with reasonable efficiency. Furthermore, we have developed methods to image pHluorin-based probes with high temporal resolution, the analysis of which allows for the resolution of exocytosis at the level of an individual synaptic vesicle.

2. Materials

2.1. Primary Culture of Hippocampal Neurons

1. Cover slips (16 mm diameter, thickness 1, BDH/VWR 406/0189/2); Poly-D-lysine hydrobromide (70,000–150,000 MW, Sigma P0899).
2. Laminin (1 mg/mL, Sigma L2020). Dissection medium: 490 mL EBSS (no calcium, magnesium or phenol red, Gibco 14155-048), 5 mL HEPES (1 M, pH 7.4, Sigma H0887), 5 mL penicillin-streptomycin (Gibco 15140-122). Store at 4°C. Standard cell culture plasticware: tissue culture flasks (75 cm² and 25 cm²), 6-well plates (Costar), 15-mL tubes. Culture medium A: 412.5 mL minimal essential medium (MEM; no phenol red, Gibco 51200-046), 10 mL Glucose (1 M in MEM); 5 mL penicillin-streptomycin, 5 mL sodium pyruvate (100 mM, Sigma S8636), 12.5 mL HEPES (1 M, pH 7.4), 5 mL N2 supplement (Gibco 17502-048), 50 mL horse serum (heat inactivated, Gibco 26050-070), sterile filter, store in the dark at 4°C.
3. Litter of E18 rat pups, approx six pups (12 hippocampi) are required for six plates (72 cover slips). Dissection kit: 2 × watchmakers forceps, dissection scalpel, fine scissors, angled scissors, small scissors, and small spatula; all bake-sterilized. Dissection microscope.
4. Papain PAP2 powder (Worthington PAPAIN-022).
5. Culture medium B: 500 mL Neurobasal (no phenol red, Earle's salts added, Gibco, 12348-017); 10 mL B27 supplement (Gibco 17504-044), 1.25 mL L-glutamine (200 mM, Gibco 25030-024), 5 mL penicillin-streptomycin. Store in the dark at 4°C.

2.2. Transfection of Hippocampal Neurons

1. Culture medium B.
2. Good-quality midprep DNA of plasmids for expression (under a cytomegalovirus promoter [CMVp]) of monomeric red fluorescent protein (mRFP) or sypHy

(~ 0.5 mg/mL, A260/A280 = 1.8). Optional: chemically synthesized short, interfering RNA (siRNA, Qiagen, 40 μ M). Lipofectamine 2000 (Invitrogen, 11668-019). MEM.

2.3. Imaging pHluorin-Based Probes at Synapses

1. Normal extracellular solution (NES): 136 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.3 mM MgCl₂, 10 mM glucose, 2 mM CaCl₂, 0.01 mM 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX; Sigma) and 0.05 mM DL-2-amino-5-phosphonopentanoic acid (DL-APV; Tocris), pH 7.4.
2. Imaging chamber (Warner instruments RC-25F), customized by the attachment of two parallel platinum wires, 5 mm apart, for electric field stimulation held in place by beeswax (Fig. 2).
3. Imaging set up: Nikon Diaphot 200 microscope with a 40 \times (1.3 numerical aperture [NA]) oil immersion objective and a Xenon 100 W epifluorescence unit. The filter set for pHluorin comprises: 475AF40 excitation filter, 505DRLP dichroic mirror and 535AF45 emission filter (Omega Filters). Using these filters with no attenuation, the illuminating light leaving the objective is approx 300 mW. To visualize mRFP, the filter set comprises: 560AF55 excitation filter, 595DRLP dichroic mirror and 645AF75 emission filter (Omega Filters). Images are captured at a depth of 16-bit using a Photometrics Cascade 512B charge-coupled device (CCD) camera controlled by an Apple Macintosh G4 computer running IPLab (Scanalytics). Where required a Uniblitz VMM-D3 shutter driver controlled by a second Mac running IgorPro (Wavemetrics) is used. Stimulation is via a Grass S48 stimulator and SIU5 stimulus isolation unit.

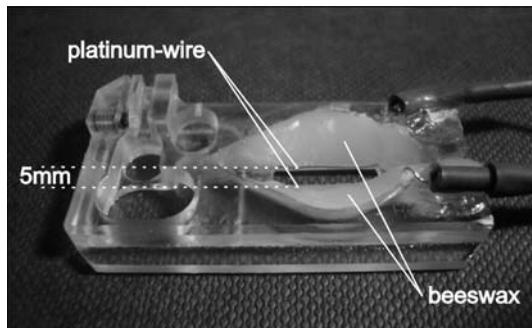


Fig. 2. The imaging chamber. Photograph of the chamber used for imaging hippocampal cultures. The chamber (RC-25F, Warner instruments) is modified by the addition of two platinum wires 5 mm apart. The wires are sealed in place by beeswax. These wires are then connected to the electrical stimulator. Cover slips of transfected neurons are attached to the underside lip of the holder and the holder is mounted into a P-1 platform (Warner instruments). Normal extracellular solution is delivered via tube into the right side of the chamber and a suction pipe withdraws solution to the left.

3. Methods

3.1. Primary Culture of Hippocampal Neurons

1. Autoclave sufficient number of cover slips, rinse in sterile ddH₂O. Put cover slips into a Petri dish with 20 mL of poly-D-lysine (50 µg/mL). Incubate at room temperature, best used within 2 wk.
2. On day 1: rinse each cover slip in ddH₂O and then place in the appropriate cell culture trays. 2 × 16 mm Cover slips are placed side by side in each well of a 6-well plate, leave for 1 h to dry in the culture hood. Apply to each cover slip 150 µL of laminin (20 µg/mL) in sterile dissection media, laminin should sit on top of the cover slip and not seep onto the well. Leave overnight in primary cell culture incubator (humidified, 37°C and 95% air/5% CO₂). Preincubate culture media A in a vented 75-cm² tissue culture flask (*see Note 1*).
3. On day 2: The dissection, trituration, and quantification steps are done in a laminar flow hood, all other culture work is done in a class II cell culture hood. Sterile procedures are used throughout. Uterus from pregnant rat is placed in 50 mL of dissection media in a 200-mL beaker on wet ice. All dissection steps are done in Petri dishes filled with cold dissection media. One by one, get the pups out by cutting the skin of the uterus with small scissors, open the membraneous sac surrounding the pup with forceps and then remove the pup (the umbilical cord will still be attached). Cut the head off and place in a Petri dish. When all the heads are removed, dissect out the brain. We find it easiest to make a small incision in the center of the nape of the neck and pull the skin and skull forward and over the brain. Once the skull is off, lift out the brain with the spatula place in media on ice. Bisect the hemispheres, peel the meninges away carefully and remove the hippocampi under a dissection microscope. It is very important to avoid contamination of the tissue with blood cells. Place dissected hippocampi in media on ice (*see Note 2*).
4. Before you dissect the last brain, get a vial of PAP2 from the fridge and add dissection media to 10 U/mL and put in 15-mL falcon tube in a 37°C water bath. Following dissection of all hippocampi, transfer to the tube. Incubate for approx 15 min, until pieces become fluffy around the edges. Let the pieces settle to the bottom of the tube (or spin for 2 min at 300g) and then remove as much digestion solution as you can. Wash with 10 mL of cold culture medium, do not disturb the pellet. Add 2 mL cold culture medium A. Triturate the pieces with two flame-polished pipets of successively smaller bore. Around five to six passes with each is normally enough, the mixture should be cloudy with no big pieces left. Use hemocytometer to quantify yield, don't count dead (granulated cells). Typically a yield is around 2.5×10^6 cells per milliliter. Diluting in cold culture medium A, a typical plating density is 0.2 to 0.25×10^6 cells per milliliter (using 150 µL per cover slip). Remove the laminin from the cover slips, but don't let it dry. Plate the neurones one tray at a time. Do not to let the cell suspension spill off the cover slip. Place the trays in the incubator. Approximately 1 or 2 h later add 2 mL of prewarmed culture medium A to each well (*see Note 3*).

5. On day 3, replace 1 mL of media with fresh prewarmed culture medium A. Then feed the cells approximately every 3 d by replacing 50% of media with prewarmed culture medium B.

3.2. Transfection of Hippocampal Neurons

1. Neurons are typically transfected after 8 d *in vitro* (DIV). Two hours prior to transfection, remove half of the culture medium and add to the well an equal volume of prewarmed culture medium B. Save the removed medium in a 25-cm² tissue culture flask and add an equal volume of fresh culture medium B, keep in incubator (*see Note 4*).
2. For one well of a 6-well plate, mix 2 μ g sypHy and 2 μ g mRFP DNA with 50 μ L MEM in a 1.5 mL tube (tube A). In tube B, mix 2 μ L of lipofectamine2000 with 50 μ L MEM, incubate at room temperature for 5 min. Add 50 μ L from tube B to tube A and mix. Incubate for 20 min in the dark at room temperature. Add mixture dropwise to the cultures, 100 μ L per well. Return cells to incubator. After 2 h, remove all media and replace with the preconditioned media kept from **step 1**. This method typically yields 2 to 20 transfected neurons per cover slip. Multiply volumes according to the number of wells required. The neurons are fed alongside the remaining cultures to keep them healthy (*see Note 5*).

3.3. Imaging pHluorin-Based Probes at Synapses

1. Imaging is carried out on neurons 14 to 21 DIV. Mount cover slips into the imaging chamber. Perfuse cells at approx 0.2 mL per minute with NES at 23 ± 2 °C using a Gilson minipuls 3 peristaltic pump. Apply suction to the opposite side of the chamber to remove surplus (*see Note 6*).
2. Find a transfected neuron by looking for mRFP expression in the RFP channel. This minimizes photobleaching of sypHy that would occur during the search for transfected neurons in the GFP channel. In the quenched (resting) state, very little sypHy fluorescence can be seen by eye (*see Note 7*).
3. We evoke action potentials using 20 mA pulses of 1 ms duration. The amount of current that is required depends on the imaging chamber and should be tested empirically by preloading untransfected cultures with fluo3-AM ester (Molecular Probes) and identifying thresholds for the Ca²⁺ signal associated with action potential firing.
4. For detection of the low fluorescence signals from single vesicles, sources of noise must be minimized. The back illuminated CCD chip in our camera was cooled to -25 °C, pixels were binned 2×2 and acquired at the lowest speed setting (5 MHz). High-quality recordings are obtained with no illumination attenuating filters or shutters and electron multiplication gain set to 2,067 with digitalization conversion gain 3 e⁻/analog-to-digital unit. The perfusion system should be turned off during image acquisition. For each cover slip, acquire two data sets. The first

consists of 10 single action potentials (APs) at 45 s intervals (2,325 images in total) and the second consists of the same 10 single APs followed by a final train of 40 APs at 20 Hz (3000 images in total). Only a subset (128×128 pixels) of the chip was used to reliably obtain all frames in data sets acquired at 5 Hz (195 ms exposure time). Each frame is triggered by a computer running IgorPro. The same program triggers the stimulation pulses (*see* **Notes 8–10**).

5. For experiments imaging responses to trains of action potentials, a “time-lapse” protocol is used. The camera exposure times and illumination are synchronized using an Uniblitz VMM-D3 shutter driver with an on and off set of 10 ms in order to minimize photobleaching. Images of sypHy (512×512 pixels) were acquired at 0.5 Hz with 250 ms of exposure at 25% illumination. In a typical experiment, 40 s (20 images) of baseline are followed by 4 or 40 stimuli at 20 Hz. Recovery is imaged for a further 120 s (240 images).

3.4. Analysis of pHluorin Signals

Data sets are analysed using the IPLab (version 3.9.4) and/or IgorPro (version 5.04 or later) software running in either MacOS X or WindowsXP. Performance is similar for both Mac and PC systems and the most important aspect for computing performance seems to be the amount of RAM. At least 1 GB is needed in either case. IPLab is a versatile and easy to use software package for image acquisition and analysis, IgorPro can do the same things as IPLab but much faster, the user interface is however more demanding and requires more time to learn.

1. Identify synapses by finding points where the fluorescence intensity increases when stimulated by 40 APs at 20 Hz. Such hot-spots were found by subtracting an average baseline signal of 5 to 20 frames immediately before stimulation to 5 to 20 images directly after.
2. Regions of interest (ROIs) are centered over each synapse. Each ROI is aligned to accommodate the largest amount of basal fluorescence surrounding the point of greatest change identified above (by eye or by custom-written scripts in IgorPro). The size of the ROIs should be as small as possible to maximize the signal-to-noise ratio, but large enough to contain both the site of fusion and endocytosis, otherwise the fluorescence measurements will not represent the true kinetics of synaptic vesicle cycling. SynaptopHluorin is very mobile and mixes with a general membrane pool after vesicle fusion (**12,13**). To allow for pHluorin movement due to this phenomenon, ROIs generally need to be bigger for synaptopHluorin than for sypHy (**6**). We suggest trying different ROI sizes and work with the smallest size that gives consistent kinetics. The authors find that ROIs of $1.6 \times 1.6 \mu\text{m}$ or more work fine for sypHy and twice that size for synaptopHluorin.
3. The illumination intensity required for imaging single synaptic vesicles rapidly leads to photobleaching of the unquenched surface pool of pHluorin molecules (**Fig. 3**). The data traces can be corrected by subtracting a double exponential

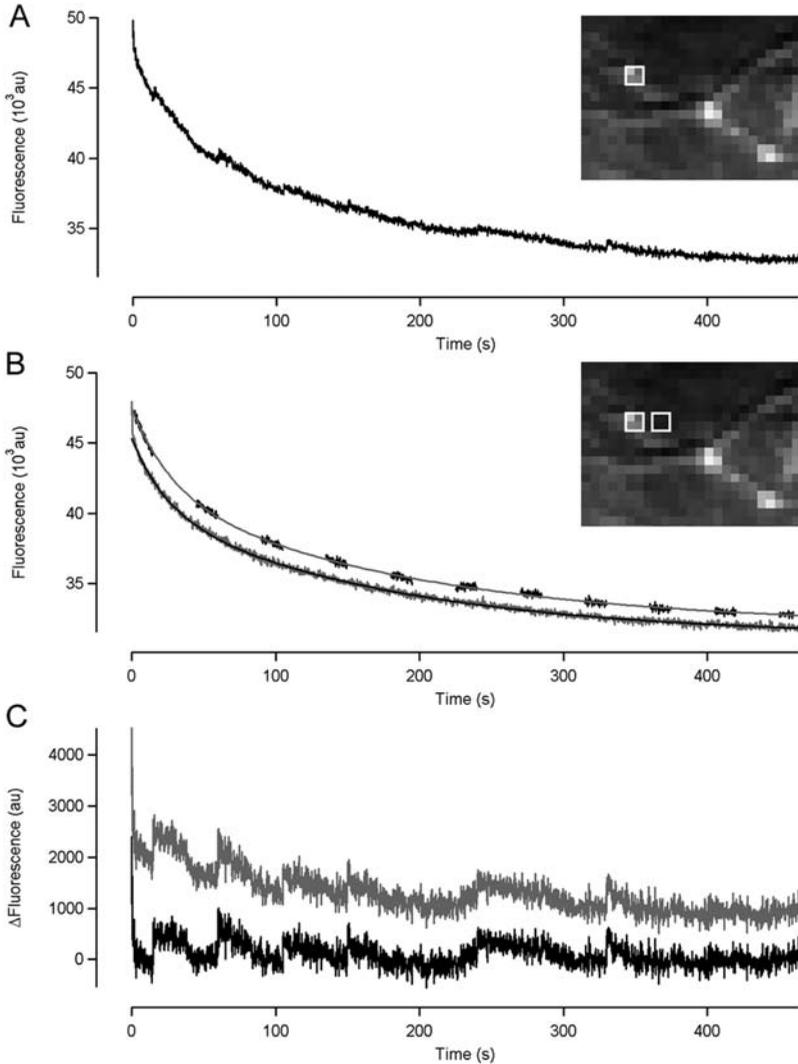


Fig. 3. Correction for photobleaching in single vesicle imaging experiments. (A) Fluorescence intensity trace (5 Hz acquisition rate) from a 2×2 pixel region of interest ROI; $1.6 \times 1.6 \mu\text{m}$, *see* inset) covering a synapse expressing sypHy. Every 45 s an action potential (AP) is evoked by field stimulation (10 times in total). (B) Bi-exponential curve fits to the fluorescence intensity trace at the synapse with 30 s following the AP blanked (grey fit to black data) or local background (black fit to gray data, *see* inset for ROI localization). (C) Traces after correction by subtracting the biexponential functions in B from the data in A. Using the function obtained at the synapse gives the trace shown in black, the gray trace is corrected using the fit to the local background.

decaying function with values from either fitting to the original trace with the 30 s immediately subsequent the stimulus blanked out or to the local background (the ROI shifted to a neighboring region without synapses, **Fig. 3**).

- Synaptic vesicle release evoked by single APs can easily be identified as rapid jumps in fluorescence time-locked to the stimulation pulses. Endocytosis is much slower than exocytosis and to reliably identify the kinetic components, many events (>100) need to be averaged before curve fitting. The fluorescence decay after a single AP can be modeled as two consecutive and irreversible reactions with first-order kinetics: endocytosis (k_e) followed by reacidification and quenching of sypHy (k_r):

$$F(t) = \frac{[A]_0}{k_r - k_e} (k_r(1 - e^{-k_e t}) - k_e(1 - e^{-k_r t}))$$

Under our experimental conditions (10 mM HEPES buffer and 23°C) the rate constant of vesicle reacidification is approx 0.25 s^{-1} . When fitted to our data, the rate constant for endocytosis is approx 0.067 s^{-1} (**6**).

- Nonphasic release of synaptic vesicles after an AP could affect the apparent time constant of the fluorescence decay. Therefore, each response to single APs should be individually examined by eye and traces containing nonphasic events discarded from further analysis.

4. Notes

- We use separate cell culture incubators for primary neuronal cultures and standard cell lines. This is to prevent possible contamination. The culturing protocol yields a mixed neuron/glia culture. Culture media are without phenol red as its color interferes with imaging experiments. Phenol red can be supplemented in one well of a plate if it is necessary to assay pH changes.
- Rat pups are used at E18. Neuronal survival is good at this age whereas at earlier ages, the meninges are troublesome to remove. Neurons from rat pups older than E18 do not survive well.
- A common cause for poor neuronal survival is the plating density of neurons being too dense or sparse. We found that changing from culture medium A to B after 3 DIV allows a wider range of densities to be used, versus leaving the cells in medium A. The dissection time should be rapid: less than 2 h between removal of the embryos and plating. The substrate is another suspicious factor if neuronal cultures are unhealthy. It is very important that laminin is aliquoted upon receipt. Aliquots are stored in a -80 C freezer. Laminin should be thawed slowly in a refrigerator for 1 h prior to use, thus preventing cross-linking of laminin molecules.

4. Transfection of neurons is a black art: we found that replacing the medium with culture media B instead of culture media A greatly improves transfection efficiency, but the reason for this is unknown. Other changes were found to improve transfection, such as incubating lipofectamine-DNA complexes in the dark, they remain in our protocol more out of superstition than a definite scientific requirement.
5. To interfere with molecules involved in synaptic vesicle cycling. Plasmids for expression of dominant-negative constructs that are mRFP-tagged can be co-transfected with sypHy, or siRNAs can be co-transfected along with mRFP and sypHy. For co-transfection with siRNA, in addition to tubes A and B, also prepare tube C, with 12 μL of 40 μM siRNA and 50 μL Neurobasal. Prepare tube D with 4 μL lipofectamine2000 and 50 μL neurobasal. Add 50 μL from tube D to tube C. Following incubations as above, add 100 μl of AB mix and 100 μL of CD mix to the well. Either keep the mixture with the cells or replace medium after 24 h. Transfections were on 11 DIV neurons and experiments performed at 72 h post-transfection.
6. When modifying the chamber the wires are placed 5 to 10 mm apart and as close to the cover slip as possible. The perfusion solution is crucial for stimulating the neurons electrically. In early experiments we used the solution described in (11), this resulted in unreliable stimulation. We believe the reason for this is culture-dependent as a different culturing protocol is used by this group. Vacuum-grease is applied to the edges of the cover slip and chamber to improve the seal between the glass and the chamber/holder (Fig. 2).
7. For the design of sypHy, we found that it was essential to position the pFluorin into the second intravesicular loop of synaptophysin, in between two cysteine residues that potentially form a disulphide bridge (14). Attempts to fuse pFluorin to the N-terminus or the C-terminus of synaptophysin did not target the pFluorin probe into synaptic vesicles. A precursor version of sypHy that lacked the fourth transmembrane domain and the C-terminus, was also incorrectly targeted. On the other hand a sypHy-double version with two pFluorin probes in tandem inserted into the second intravesicular loop showed brighter fluorescence at the synapses before stimulation but did not result in a better signal-to-noise ratio. We therefore opted to work with the original sypHy construct.
8. We do not reduce background noise by photobleaching synaptopFluorin or sypHy before applying test stimuli, as suggested in (10). In our hands, extensive pre-bleaching damages the cells.
9. Although cooling the CCD more than -25°C would decrease the dark current, spurious charge electrons become more frequent and skew the background noise distribution toward more electrons.
10. Our imaging setup is on a vibration-isolation table (VH3036-OPT, Newport) covered by a dark Faraday cage. When imaging single vesicles, experiments are carried out with no room lighting and with computer monitors switched off to reduce ambient light. The cage is fitted with a heavy black cloth curtain to prevent any remaining light leaking in. An electric fan is essential to circulate air to reduce the build up of heat generated from the camera and lamp in the cage.

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