Video Article

An Optical Assay for Synaptic Vesicle Recycling in Cultured Neurons Overexpressing Presynaptic Proteins

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Abstract

At active presynaptic nerve terminals, synaptic vesicles undergo cycles of exo- and endocytosis. During recycling, the luminal domains of SV transmembrane proteins become exposed at the cell surface. One of these proteins is Synaptotagmin-1 (Syt1). An antibody directed against the luminal domain of Syt1, once added to the culture medium, is taken up during the exo-endocytotic cycle. This uptake is proportional to the amount of SV recycling and can be quantified through immunofluorescence. Here, we combine Syt1 antibody uptake with double transfection of cultured hippocampal neurons. This allows us to (1) localize presynaptic sites based on expression of recombinant presynaptic marker Synaptophysin, (2) determine their functionality using Syt1 uptake, and (3) characterize the targeting and effects of a protein of interest, GFP-Rogdi.

Video Link

The video component of this article can be found at https://www.jove.com/video/58043/

Introduction

Studying synaptic vesicle recycling is important in determining how presynaptic properties change, either during synaptic plasticity or in response to perturbation of synaptic function. Studying Synaptotagmin-1 (Syt1) antibody uptake provides one method of measuring the amount of SV recycling. Syt1 is a SV-associated protein that acts as a Ca^{2+} sensor and is necessary for exocytotic release of the neurotransmitter[1,2]. It is a transmembrane protein with a C-terminal cytoplasmic domain outside the SV and an N-terminal luminal domain inside the SV[3]. During exocytosis, the luminal domain of Syt1 becomes exposed to the external medium. To this external medium, we add antibodies directed against the cytoplasmic domain, which becomes internalized during endocytosis. These antibodies can be either pre-conjugated with fluorophores or immunostained with secondary antibodies[4,5,6,7]. The fluorescence intensity of the resulting immunosignal is proportional to the amount of SV recycling. This approach can be used to determine both constitutive and depolarization-induced SV recycling[6,8].

Syt1 uptake assays can be performed after virus-mediated gene transfer to virtually all cells in the dish or after sparse transfection of a small number of cells. Our method combines the assay with sparse double transfection of primary hippocampal neurons using calcium phosphate[9]. We use a recombinant marker protein known to accumulate at presynapses, fluorescently tagged Synaptophysin, to locate presynaptic terminals and overexpress our protein of interest, Rogdi. This allows us to test whether or not Rogdi targets functional synapses and affects SV recycling. The gene encoding Rogdi was originally identified in a screen for Drosophila mutants characterized by impaired memory[10]. In humans, mutations in the Rogdi gene cause a rare and devastating disease called Kohlschütter-Tönz syndrome. Patients suffer from dental enamel malformations, pharmacoresistant epilepsy, and psychomotor delays; however, the subcellular localization of the gene product remained elusive[11]. Thus, the Syt1 uptake assay provided key evidence for the localization of GFP-tagged Rogdi at functional synapses[9].

This uptake technique has several benefits. First, SV recycling can be observed both in real time by performing live imaging[7,12], and after fixation[6,9] by measuring the fluorescence intensity of the Syt1 fluorescence label. Additionally, several Syt1 antibody variants have been developed. There are untagged variants that can be labeled with a secondary antibody following a standard immunostaining protocol after fixation, and pre-conjugated variants with a fluorescence label already attached. Finally, antibody-based fluorescence is advantageous due to the large selection of commercially available secondary or conjugated dyes that can be used.

When fixing and immunostaining the neurons, it is also possible to stain for additional proteins and perform colocalization analysis. This can help determine where they are located in relation to recycling SVs. The intensity of the fluorescence label is the direct measure of the amount of SV recycling. In addition, the antibodies selectively label Syt1-containing structures, resulting in high specificity and little background fluorescence[4]. Different stimulation protocols can also be used, such as depolarization buffers or electric stimulation protocols[9,12,13,14]. However, basal SV recycling can be measured without stimulating the neuronal cultures[15].

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Our method specifically addresses Syt1 antibody uptake in double-transfected neurons with secondary antibody immunolabeling after fixation. However, we refer to all routinely used variants of the assay in our discussion to give viewers an opportunity to adapt the protocol to specific needs.

Protocol

No studies with live animals were conducted. Experiments involving euthanized animals to obtain cell cultures were approved by the local animal protection authorities (Tierschutzkommission der Universitätsmedizin Göttingen) under the approval number T10/30. The experiments were conducted with the approved protocols.

1. Primary Hippocampal Cell Culture

1. Prepare the dissociated cell culture of the rat hippocampus on embryonic day 19[16,17]. Plate the cells on 12 mm coverslips coated with polyethyleneimine (PEI) in 24-well dishes at a density of 50,000 - 60,000 cells/well. Check the density using a cell counting chamber and phase contrast optics.
2. Culture the neurons for 3 days (day *in vitro* (DIV) 3) in a 24-well plate in the incubator at 37 °C with 5% CO_2.
3. Assess the coverslips for indicators of cell health using transmitted light microscopy (*e.g.,* phase contrast optics at a magnification of 10 - 20X). Check for the following indicators of good health: a clear phase contrast halo, neurites without beaded structures, and no soma clustering or neurite bundling.

2. Transfection

Note: The following protocol refers to a double-transfection for 3 wells. However, the protocol works best when amounts sufficient for 4 wells are prepared.

1. Prepare 500 mL of transfection buffer (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM glucose, 42 mM HEPES) in an Erlenmeyer flask.
    1. Dissolve 8.0 g of NaCl, 0.37 g of KCl, 0.095 g of Na_2HPO_4, 1.35 g of glucose, and 5.0 g of HEPES in 400 mL of distilled water in an Erlenmeyer flask.
    2. Adjust the pH to 6.95 with 1 M NaOH using a pH meter.
    3. Adjust the volume with distilled water to 500 mL and check the pH using a pH meter.
    4. Make 20 - 30 mL aliquots of transfection buffer with the following pH values by pipetting 1 M NaOH to the transfection buffer: 6.96, 6.97, 6.98, 6.99, 7.00, 7.01, 7.02, 7.03, 7.04, 7.05, 7.06, 7.07, 7.08, 7.09, 7.11.
    Note: The pH of the transfection buffer is crucial for the transfection efficacy.
    5. To test which transfection buffer leads to the highest number of transfected cells, test each pH value from 6.96 to 7.11. Use the transfection method described in 2.2 - 2.11 and a validated plasmid expressing GFP. Determine the number of transfected cells per coverslip for every transfection buffer pH value to assess which buffer works the best.
    6. Aliquot the buffer with the highest transfection efficiency into 2 mL microcentrifuge tubes Freeze and store the tubes at -20 °C.
2. Pre-warm the reduced serum medium, cell culture medium, and distilled water to 37 °C in the water bath.
3. Prepare the transfection mix in a 1.5 mL microcentrifuge tube. Work under the laminar flow hood to ensure sterile working conditions.
    1. Mix 7.5 µL of 2 M calcium chloride with 4 µg of each endotoxin-free DNA (Synaptophysin-mOrange and mGFP/GFP-Rogdi). Add water to reach a total volume of 60 mL in a 1.5 mL microcentrifuge tube.
    2. Add 60 mL of transfection buffer to the mix. To obtain the best results, add the transfection buffer dropwise while shaking the DNA-mix gently on the vortex.
    3. Incubate at room temperature (RT) for 20 minutes. Avoid shaking the incubation tube during the incubation time by placing the tube next to the laminar flow hood.
4. Under the laminar flow hood, remove the cell culture medium (“conditioned medium”) from the wells using a 1000 mL pipet and store it in a separate container in the incubator.
5. Add 500 mL of reduced serum medium to each well. Incubate the cells at 37 °C and 5% CO_2 until the 20 minute incubation period (step 2.3.3) is over.
6. Add 30 mL of transfection mix to each well by pipetting several drops. Discard the residue at the bottom of the tube.
7. After all the wells have been supplied with transfection mix, gently shake the 24-well plate to ensure distribution of the transfection mix in the medium.
8. Incubate the wells for 60 minutes at 37 °C and 5% CO_2.
9. Remove and discard the transfection mix and wash it three times with cell culture medium. Add 1 mL of cell culture medium to each well and incubate them for 30 seconds at RT. Remove 750 mL of medium and add the same amount of fresh medium. Repeat this three times.
Note: The washing step is critical. Keep the time that each well has no medium at a minimum (*i.e.*, remove and replace well-by-well) and add the washing medium gently.
10. Remove and discard the cell culture medium and add 450 mL of the conditioned medium well-by-well.
11. Let the neurons mature in the incubator at 37 °C and 5% CO_2 to DIV 10.

3. Stimulation and Syt1 Uptake

Note: The following protocol applies the uptake to 3 wells. For depolarization of any other number of wells, adjust the amounts accordingly.

1. Prepare 50 mL of 10x depolarization buffer (640 mM NaCl, 700 mM KCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 300 mM glucose, 200 mM HEPES, pH 7.4) in an Erlenmeyer flask.

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Note: Depolarization buffer can be kept at 4 °C for several weeks. If a non-depolarizing solution is also used, , prepare a 10x Tyrode's solution consisting of 1290 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 300 mM Glucose, 200 mM HEPES pH 7.4 in order to compare stimulation-induced recycling with spontaneous recycling. After dilution to 1x, add 1 µM of Tetrodotoxin before use to block action potential generation.

1. Dissolve 1.87 g of NaCl, 2.61 g of KCl, 0.1 g of $MgCl_2$-$6H_20$, 0.15 g of $CaCl_2$-$2H_2O$ and 3.0 g of glucose-1 H_2O and 2.38 g of HEPES in 50 mL of distilled water in an Erlenmeyer flask. Adjust the pH with NaOH and sterile-filter the solution. Dilute the buffer 1:10 in distilled water to achieve a 1x concentration.

2. Prepare 4% paraformaldehyde (PFA) in 1x PBS (pH 7.4) for fixation after stimulation.
    1. For 500 mL of 4% PFA in 1x PBS, dissolve 20 g of paraformaldehyde in 450 mL of distilled H_2O.
    Note: Heating the solution may speed up dissolving, but do not heat the solution over 70 °C, as the PFA may disintegrate.
    Caution: PFA is toxic, potentially carcinogenic, and teratogenic. Wear gloves when working with PFA, work under the fume hood, and avoid ingestion.
    2. Let the solution cool to RT and add 50 mL of 10x PBS stock solution. Adjust the pH to 7.4 with NaOH/HCl using a pH meter.
3. Pre-warm 600 mL of 1x depolarization buffer and 10 mL of cell culture medium to 37 °C in the water bath.
4. Add 1 mL of mouse anti-Syt1 antibody (clone 604.2) to the 1x depolarization buffer and vortex for 10 seconds.
5. Remove and discard the cell culture medium from the cells. Add 200 mL of the depolarization-antibody mix to each well and incubate for 5 minutes at 37 °C and 5% CO_2 in the incubator.
6. Remove and discard the depolarization-antibody mix and wash it three times with cell culture medium. Add 1 mL of cell culture medium to each well and incubate for 30 seconds at RT. Remove 750 mL of medium and add the same amount of fresh medium. Repeat three times.
7. Remove and discard the cell culture medium and add 300 mL of 4% PFA in 1x PBS. Incubate for 20 minutes at 4 °C.
8. Wash three times for 5 minutes each with 1x PBS.
Note: The protocol can be paused here.

4. Immunocytochemistry

1. Prepare 50 mL of blocking buffer.
Note: Blocking buffer can be kept at -20 °C for several months.
    1. Dissolve 2.5 g of sucrose and 1 g of bovine serum albumin (BSA) in 5 mL of 10x PBS stock solution. Add 1.5 mL of 10% detergent stock solution. Stir the solution until all the components have properly dissolved and add distilled H_2O until reaching a final volume of 50 mL. Aliquot the solution and freeze the aliquots for storage.

2. Dilute the secondary, fluorophore-coupled antibody (directed against the primary Syt1-antibody species) in 200 mL of blocking buffer in each well at a dilution of 1:1000.
3. Remove and discard the 1x PBS from each well containing a coverslip.
4. Add 200 mL of blocking buffer-antibody mix to each well and incubate for 60 minutes at RT.
Caution: Because the secondary antibodies are light-sensitive, all steps moving forward must be performed in the dark.
5. After incubation, wash the cells three times for 5 minutes with 1 mL of 1x PBS.
6. Embed the coverslips on microscope slides with the embedding medium.
    1. Add a 7 mL drop of embedding medium onto the microscope slide. Remove the coverslip from the 24-well plate by lifting it with a syringe and grabbing it with forceps.
    Caution: Cells on the surface of the coverslip are easily damaged, so forceps must be handled with care.
    2. Dip the coverslip into distilled water to remove the PBS and dry it carefully by touching one edge to a soft tissue.
    3. Flip the coverslip onto the embedding medium droplet, so that the surface carrying the cells faces the microscope slide, thereby embedding the cells into the embedding medium.

7. Leave the slides to dry under the hood for 1 - 2 h (cover them to avoid light exposure) and store them in a microscope slide box at 4 °C.
Note: The protocol can be paused here.

5. Microscopic Analysis

1. After the coverslips dry, place them under the microscope with the objective and camera.
2. Adjust the exposure time for every channel so that few pixels are overexposed to ensure maximum distribution of grey values.
Note: While the exposure time may vary between the channels, it should be constant for one channel to ensure comparability between the coverslips.
3. Acquire multi-channel images for 10 regions of interest (ROIs) per coverslip. Check that the ROI contains axonal processes from a transfected neuron by checking GFP-fluorescence, which should be punctate.

6. Statistical Analysis

1. Export the images as .tif files. Load the images into OpenView[18] by clicking **File** | **Load image file**.
2. Choose the Synaptophysin-mOrange image as channel 1, the Rogdi-GFP/mGFP image as channel 2, and the Alexa647 fluorescence image as channel 3.
3. Threshold the ROIs.
    1. Click **Analysis** | **Place area over puncta**. Choose **Threshold** and **Delta intensity** values so that upon visual inspection, diffuse fluorescence is excluded, leaving only punctate signals in the image of channel 1 (representing Synaptophysin-mOrange fluorescence). Keep the same threshold for all images.

4. Transfer these ROIs to the corresponding channel 2 (GFP-Rogdi/mGFP fluorescence) and 3 (Syt1-fluorescence) of each coverslip area by clicking **Execute now**.
   Note: The ROIs should only be considered if the cell is double-transfected. In this method, mOrange- and GFP-fluorescence should be clearly visible.
5. Save the data into the analysis log editor by clicking **Log data**.
6. Open the analysis log editor under the **Windows** tab and copy the values for each channel. Paste the values for the separate channels into a spreadsheet.
7. Determine the average fluorescence intensity of the Syt1-channel at the ROIs in both transfection conditions (GFP-Rogdi and mGFP).
8. Apply appropriate statistical tests such as Student's *t-test* to determine significant differences.

Representative Results

An expected result of this approach is locating approximately 50 double-transfected neurons per coverslip at a density of 50,000 neurons per well. The axon of each neuron is expected to show multiple hotspots of fluorescently-tagged Synaptophysin accumulation, indicating clusters ofSVs. At functional presynaptic sites, the recombinant Synaptophysin signal colocalizes with punctate Syt1 fluorescence. Using double transfection, either GFP-Rogdi as the protein of interest (**Figure 1**) or mGFP as the control is co-expressed with recombinant Synaptophysin.

In this experiment, we analyze SV recycling at presynaptic sites in neurons expressing either GFP-Rogdi or mGFP. If the control protein, mGFP, is homogeneously distributed throughout the whole cell, but its influence at synaptic sites still needs to be studied, then it is necessary to co-transfect a second protein that is presynaptically enriched, fluorescently tagged Synaptophysin.

As described earlier, cells undergo depolarization-induced transmitter release. As a result, functional synapses take up Syt1 antibodies added to the medium. After immunolabeling the Syt1 antibody with a fluorescently labeled secondary antibody, the extent of Syt1 uptake is finally quantified using the immunosignal (**Figure 2**).

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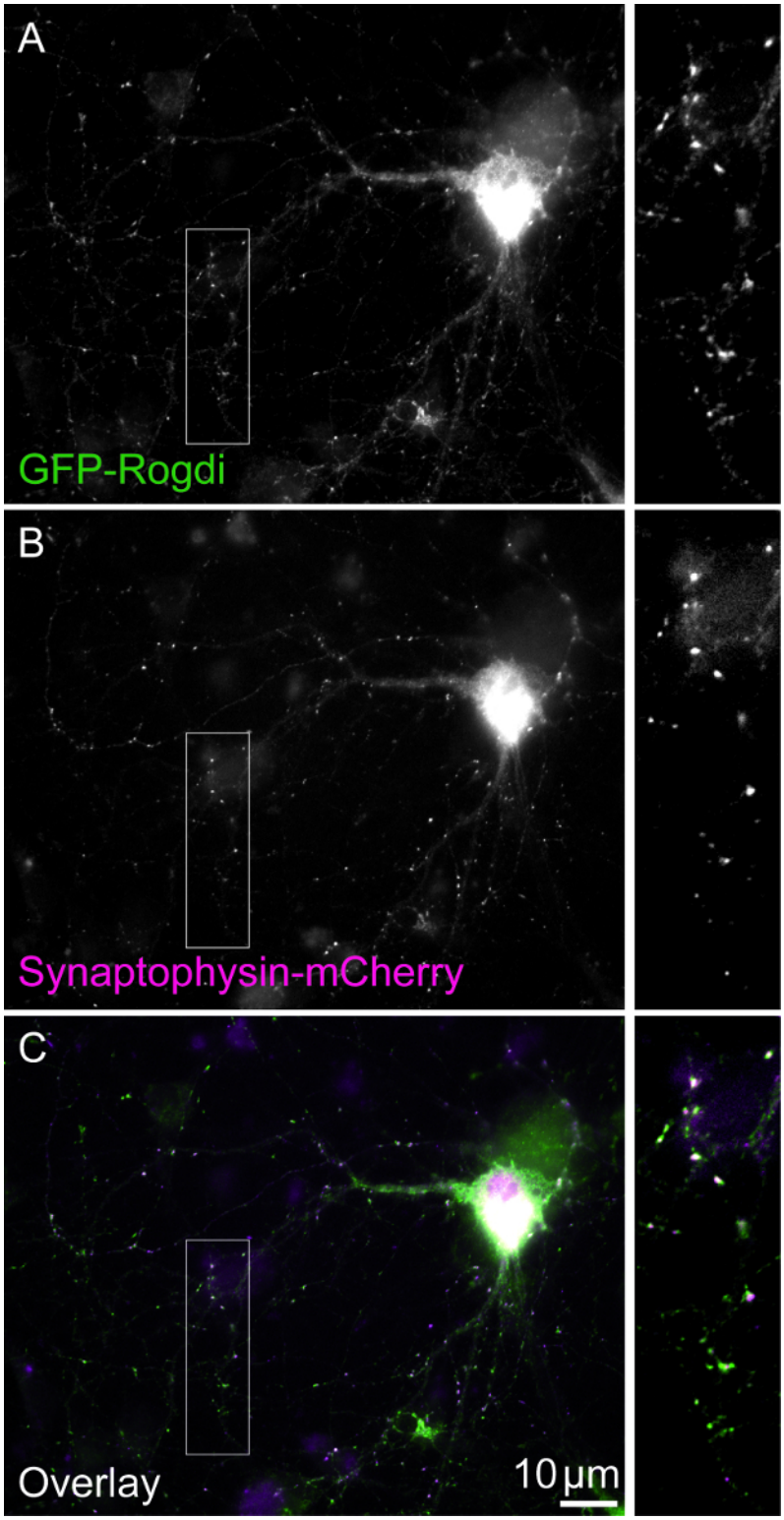

Figure 1. Double-transfected cell. The field of view shows a double-transfected cell expressing GFP-Rogdi (A) and Synaptophysin-mCherry (B). Synaptophysin is an abundant synaptic vesicle protein. Its recombinant variant can be used for labeling synaptic boutons. The localization pattern of GFP-Rogdi resembles that of Synaptophysin-mCherry (C). Scale bars = 10 µm. Boxes show 2.5X magnification. Please click here to view a larger version of this figure.

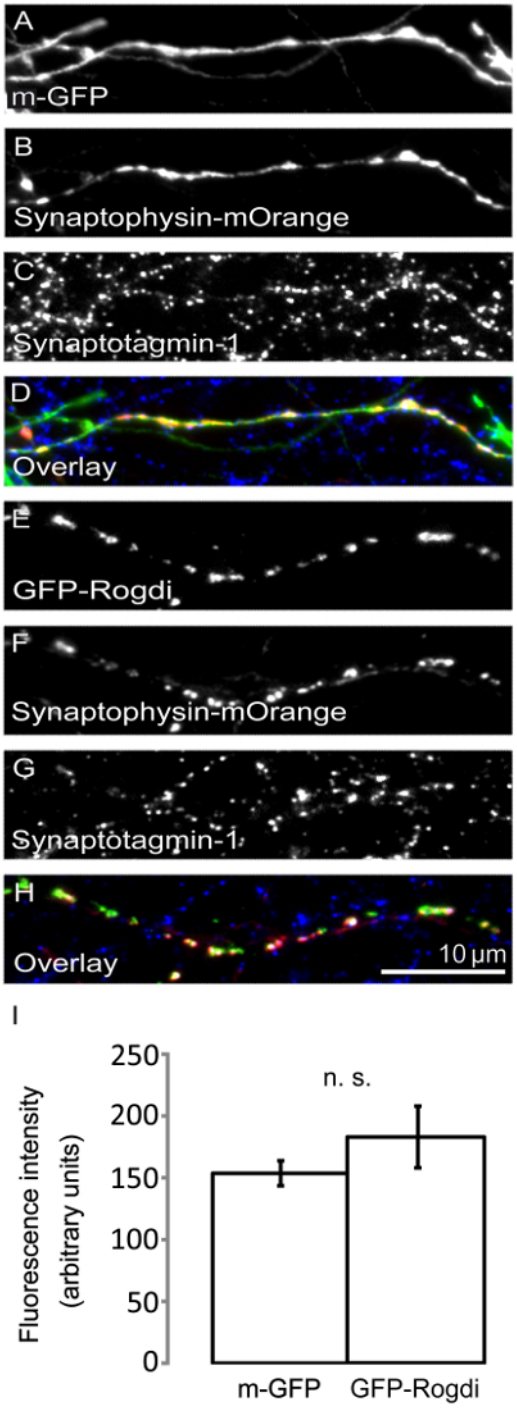

Figure 2. GFP-Rogdi at functional synapses. Live Syt1 uptake was performed in double-transfected cells expressing either mGFP (green) and Synaptophysin-mOrange (red; A-D) or GFP-Rogdi (green) and Synaptophysin-mOrange (red; E-H). Syt1 antibody uptake was performed using a mouse monoclonal antibody directed against the luminal domain of Syt1. After PFA fixation, cells were stained with rabbit anti-GFP antibody. Secondary antibodies (anti-rabbit Alexa 488 and anti-mouse Alexa 647) were added to detect GFP or GFP-Rogdi and Syt1, respectively. Autofluorescence of Synaptophysin-mOrange was used to detect presynaptic terminals (B and F). Punctate Syt1 immunofluorescence indicates sites of vesicle recycling (blue; C and G). Note that the majority of Syt1-labeled synapses are localized to non-transfected neurons. GFP-Rogdi was targeted to active synapses and did not change synaptic vesicle recycling (I). 3 independent culture experiments were performed (N = 3). At least 3 coverslips from each culture (total of 10) were used for analysis. Finally, 3 areas per coverslip were analyzed (n=30). Student´s *t-test* showed no significant difference. Scale bars = 10 µm. Error bars represent the standard error of the mean (S.E.M.). "N.s." indicates no significance. This figure has been modified from Riemann *et al.*[9] Please click here to view a larger version of this figure.

Discussion

There are three assays routinely used to study synaptic vesicle (SV) recycling. The first two include the use of a) fluorescent styryl dyes such as FM1-43 (which incorporate into membranes, are taken up into organelles during endocytosis, and are released after exocytosis); and b) fluorescently tagged recombinant SV proteins (which, upon overexpression, incorporate into the proteinaceous recycling machinery). If the attached fluorophores change their fluorescence depending on the pH, they can be used to monitor changes between the acidic interior of a SV and the pH of the extracellular medium. The recombinant proteins tagged with a pH-sensitive variant of GFP are called Phluorins. The two assays discussed have both been featured previously[19,20], and the pros and cons of each have also been reviewed[21].

Here, we describe a third, well-established method[4,5,6,7,9,14]. To test SV recycling, we take advantage of the fact that the luminal domain of vesicle-associated protein Synaptotagmin-1 (Syt1) becomes exposed to the cell surface upon exocytosis. First, we add an antibody directed against the luminal domain to the culture medium, which is then taken up by the vesicle during SV recycling. This antibody uptake is visualized and quantified by immunofluorescence. Alternatively, a directly labeled Syt1-antibody can be used. The label can be pH-independent, reporting localization of the Syt1-antibody both inside and on the external surface of SVs, or pH-dependent, reporting localization based on fluorescence changes. We also describe the combination of this SV recycling assay with double transfection of cultured neurons to test whether or not a protein of interest, GFP-Rogdi, targets functional synapses and affects SV recycling. GFP-Rogdi is specific to this approach; however, the same questions can be addressed for any protein of interest.

This assay was first introduced in 1992 to monitor the spontaneous recycling of SVs[4] and was further developed by the same group to monitor evoked SV recycling[12]. Their experiments established that Syt1 uptake is sensitive to clostridial neurotoxins, which block SV exocytosis[12]. They also showed that stimulation of cultures by depolarization at 37 °C enhances the internalization of Syt1 antibodies compared to 1) cultures that are kept on ice, which prevents most endocytosis, and to 2) cultures incubated at 37 °C without stimulation, which allows for spontaneous recycling but does not evoke recycling[12,22]. The assay can readily compare SV recycling between two conditions, such as with and without a molecule of interest. In particular, this protocol focuses on comparing Syt1 antibody uptake in the presence versus the absence of a certain overexpressed protein. The protocol can be expanded if the relative contributions of surface-binding, spontaneous recycling and evoked recycling need to be assessed. For example, incubating neurons on ice without stimulation prevents endocytosis, revealing any surface binding of the Syt1 antibody. Incubating the neurons at 37 °C in basal solution with Tetrodotoxin prevents action potential propagation, revealing any spontaneous recycling. Thus, incubating neurons at 37 °C under stimulating conditions can reveal the extent of evoked SV recycling.

Live cells are sensitive to ideal physiological conditions; therefore, maintenance, transfection, and depolarization buffers should always be tested for physiological parameters including pH and temperature. While depolarization buffers can be passed through sterile filters after the pH has been adjusted, transfection buffers are sensitive to sterile filtering. Therefore, we adjust its pH, keep it frozen until use, and spin it in a table-top centrifuge to pellet any bacteria. For transfection, it is vital to mix the buffer adequately to ensure association of the plasmid with the calcium phosphate crystals. Calcium phosphate transfection works best on day *in vitro* (DIV) 2 - 4. We use tagged Synaptophysin, a SV transmembrane protein, as a marker for identifying presynaptic terminals in the transfected neurons. Tagged versions of other SV proteins such as SV2, Synapsin, and VAMP/Synaptobrevin or active zone molecules such as Bassoon, RIM, Munc13-1, and CAST can also be used.

Both mCherry and mOrange emit red fluorescence. Although mCherry is brighter than mOrange, it has greater emission at long wavelengths than mOrange. Therefore, for triple fluorescence imaging with GFP, a red dye, and a dye emitting in the 700 nm range, mOrange is a better option than mCherry. For double fluorescence with GFP and a red dye, mCherry may be favorable since it is the brighter dye. To guarantee sparse transfection, the transfection mix should not be incubated for longer than 60 minutes. It is also important that incubation in a depolarizing buffer is the right length of time to ensure exocytosis of all vesicles. However, extended exposure to the depolarizing buffer must be avoided. During image acquisition, it is also essential to apply the same exposure times and light intensities to ensure quantifiable comparison between different sets of experiments.

Finally, during image analysis, our protocol includes several considerations. For one area of interest showing recombinant Synaptophysin puncta, the lower-intensity threshold is set so that diffuse fluorescence is excluded. Next, this threshold is tested on other areas and coverslips to observe similar inclusion of punctate signals and exclusion of diffuse fluorescence. Once an appropriate threshold is identified, it is applied to all images, producing punctate regions of interest (ROIs) in the recombinant Synaptophysin (tagged with mCherry or mOrange) channel. Lastly, Syt1 intensity is determined for the ROIs[9].

Our approach is optimized for sparse transfection. We analyze presynaptic function in a complex setting where there is SV recycling in axons of transfected neurons surrounded by untransfected neurons. In this set-up, double-transfection allows us to manipulate neuronal function and locate the presynaptic terminals of the transfected neurons. Transfected presynaptic neurons that contact untransfected postsynaptic neurons is an important factor in assessing a presynaptic neuron's effects on SV recycling. The setting can also be easily adapted to test whether or not a postsynaptic manipulation causes presynaptic changes. In this case, sparse transfection of neurons with a protein that is targeted to postsynaptic sites will localize the postsynaptic sites of the transfected neurons, and Syt1 uptake can be determined at synaptic boutons of untransfected neurons.

When sparse transfection is not necessary, different transfection protocols can be applied. For example, transfection with Lipofectamine on DIV 7 - 8 results in higher transfection rates, but it also entails stronger expression in each transfected cell[9]. Furthermore, viral transduction can result in expression in nearly 100% of cultured neurons[14,23,24]. In experiments aimed at monitoring SV recycling, transfection can be performed for a single protein or left out completely. For example, when overall synaptic recycling is compared between neurons from wildtype and genetically modified mice, labeling individual neurons by transfection is not necessary. Additionally, this enables immunolabeling of endogenous proteins. If the transfection fails, it is important to test the transfection buffer with a validated plasmid or use a buffer with a different pH (see *Preparation of transfection buffer*). Using endotoxin free DNA preparations also appears to be crucial. Immunostaining internalized Syt1 antibodies works in both PFA-fixed and methanol-fixed samples. It should also be noted that GFP and RFP autofluorescence is lost after methanol fixation. If GFP or RFP need to be localized in methanol-fixed cells, these proteins must be immunolabeled. If Syt1 uptake fails, depolarization time and K^+ concentration in the depolarization buffer should be changed accordingly. Many protocol variations have been published, with depolarization

times ranging from 90 s[25] to 60 min[12], and with K^+ concentrations of 45 mM, 50mM, 70 mM, and 110 mM[6,25,26,27]. When recurrent activity must be prevented, glutamate receptor blockers can also be added during depolarization. Finally, while high K^+ depolarization is thought to be the strongest stimulus, action potentials can induce SV recycling through more physiological stimuli[22,28]. Differences in Syt1 antibody uptake efficacy might occur in rat and mouse cultures. In our method, the monoclonal mouse anti-Syt1 clone 604.2 (RRID:AB_993036) produces stronger staining in rat cultures than in mouse cultures, while the polyclonal rabbit anti-Syt1 antibody (RRID:AB_11042457) produces stronger staining in mouse cultures, but several notes of caution are discussed below.

Several caveats regarding the use of anti-Synaptotagmin antibodies for uptake into SVs should be considered. First, N-glycosylation of asparagine 24 of Syt1 promotes the endocytotic uptake of the protein[29,30]. Asparagine 24 is part of the luminal domain of Syt1. The mouse and rabbit antibodies that we use are directed against amino acids 1 - 12 and 1 - 8, respectively, of Syt1. While their epitopes do not overlap with the N-glycosylation site, steric hindrance or, conversely, an induction of uptake by antibody binding cannot be excluded. Mutation of the N-glycosylation site in Syt1 does not change the kinetics of endocytosis and the targeting of Syt1 to SVs. It increases the fraction of Syt1 remaining on the outer plasma membrane surface when endocytosis is induced by weak stimuli. When strong stimuli, such as high K^+ depolarization, are applied, no reduction in Syt1-uptake is observed[30]. Overall, if the antibodies interfered with the N-glycosylation site in some way they might cause a change in SV endocytosis compared to antibody-free conditions, but this change should be similar for the experiment (in our case the overexpression of GFP-Rogdi) and the control (in our case the expression of GFP). Additionally, polyclonal antibodies may be more likely to induce steric problems and crosslinking than monoclonal antibodies, because a larger copy number of antibodies binds to the epitope. We are not aware of systematic studies comparing the available antibodies with each other; however, some studies have addressed the validity of certain antibodies. For example, when SV recycling kinetics were probed using the monoclonal mouse anti-Syt1 antibody clone 604.2 and Synaptophluorin-fluorescence changes in a direct comparison, similar results were obtained, validating the use of this antibody[28]. Loading SVs with polyclonal rabbit Syt1 antibodies and recording synaptic transmission electrophysiologically revealed that the loaded synapses had reduced synaptic transmission in a way reminiscent of loss-of-function mutants of Syt1. Therefore, these polyclonal antibodies do affect synaptic transmission. The actual uptake revealed both spontaneous and evoked recycling, suggesting that a polyclonal antibody is suitable to monitor one round of SV recycling, but caution should be taken when interpreting results after uptake of these antibodies[31].

Three assays are routinely used to study SV recycling, which include membrane labelling with styryl dyes, overexpression of Phluorins, and the Syt1-antibody uptake performed here. All three assays can be used to determine endocytotic and exocytotic legs of SV recycling. While all three assays have powerful benefits, each also has a principle caveat. FM dyes label the entire cell surface and are taken up into the cell with all recycling membranes. Phluorins must be introduced by overexpression. Syt1-antibodies label the endogenous protein, but some antibodies may affect its function under certain conditions. Each caveat can, however, be appropriately addressed[21].

Overall, the Syt1 antibody uptake assay has served various purposes. First, it was used to selectively label SVs undergoing spontaneous or evoked recycling, respectively[28,31,32,33]. Second, it was used to determine the effect of a drug or protein on the extent of SV recycling; for example, to show that BDNF enhances both spontaneous and evoked SV recycling[6]. Third, it was used to determine the number of synapses with recycling SVs as a percentage of the total number of morphologically defined synapses[34,35]. This revealed that overexpressing the postsynaptic cell-adhesion molecule Neuroligin-1 increases the percentage of synapses with recycling SVs[25] and that reducing the expressing of AMPA-type glutamate receptors decreases the percentage of synapses with recycling SVs, increasing the percentage of presynaptically silent synapses[36]. Additionally, the assay was used to analyze the mobility of labelled SVs[27] and to determine the localization of Syt1 using nanoscopy[13,22,33]. Finally, this method is still used to test whether or not certain proteins localize to functional synapses[9]. The Syt1 antibody can also be used for long-term studies, where the antibody remains in the neurons for days following uptake[37]. This can be combined with a second round of uptake using a separately labeled Syt1 antibody or an antibody directed against the luminal domain of a different SV protein. This allows testing of which SVs recycled initially and which recycle by the end of an experiment. It would be interesting to adapt this assay to more intact model systems, such as acute slices and organotypic slice cultures. In principle, the uptake assay allows comparison of tissue from wildtype and genetically modified animals or may be combined with viral delivery or biolistics, *i.e.*, a "gene gun," to manipulate protein expression in these model systems. In such systems, the influence of certain perturbations on presynaptic machinery, monitored by the uptake assay, and network dynamics can be studied simultaneously.

Disclosures

The authors have nothing to disclose.

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