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Imaging Ca^{2+}-Triggered Exocytosis of Single Secretory Granules on Plasma Membrane Lawns from Neuroendocrine Cells

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Summary This cell-free assay for exocytosis is particularly useful when spatial information about exocytotic sites and biochemical access to the plasma membrane within less than a minute is required. It is based on the study of plasma membrane lawns from secretory cells exhibiting secretory granules filled with neuropeptide Y-green fluorescent protein (NPY-GFP). The sample is prepared by subjecting NPY-GFP-expressing cells to a brief ultrasound pulse, leaving behind a basal, flat plasma membrane with fluorescent attached secretory organelles. These sheets can then be incubated in defined solutions with the benefit that complete solution changes can be achieved in less than 1 min. Individual secretory granules are monitored in the docked state and during exocytosis by video microscopy.

Keywords Cell-free assay; dense core granules; fluorescence microscopy; GFP; peptide hormone; plasma membrane sheets; secretion.

1 Introduction

Neurotransmitter and hormone release is a highly regulated process, involving many factors that form membrane protein complexes, which facilitate the fusion of secretory organelles with the plasma membrane. The nature of these complexes implicates that they are difficult to characterize biochemically. Many methods have been developed to unravel the exocytotic cascade of biochemical reactions. These include biochemical cell-free assays, electrophysiological approaches such as amperometry and capacitance measurements, or optical detection of exocytosis. Each type of assay has its advantages and limitations

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concerning the rate at which exocytosis is monitored, the ease by which the biological sample can be manipulated biochemically, and detection of secretory organelles not only during fusion but also when transported and docked to special sites of the plasma membrane.

The cell-free assay described here provides spatial information about exocytotic sites and quick biochemical access to the plasma membrane. Mechanically removing the upper part of the cell (for review, *see Ref. 1*), the inner leaflet of the basal plasma membrane is made biochemically accessible within seconds. To this end, cells are subjected to shearing forces that are generated by a brief ultrasound pulse, leaving behind a flat, cortical membrane lawn with attached secretory granules. Granules are labeled with green fluorescent protein (GFP) by expression of a corresponding GFP-based secretory granule marker *(2)*. Such granules can be monitored by fluorescence microscopy before, during, and occasionally after exocytosis *(3)*. The experimental time window and sampling frequency are limited by the brightness of the secretory granules and the sensitivity of the camera system. In practice, taking four images per minute at diffraction-limited resolution for 15 min is feasible, thus allowing temporal and spatial resolution of individual fusion events and monitoring of biological activity occurring during a stimulus. GFP fluorescence can be well separated from fluorophores emitting in the red or far-red range, allowing for simultaneous monitoring of exocytosis and a fluorescent-labeled membrane-associated protein present at the exocytotic site *(4)*.

2 Materials

2.1 Cell Culture and Transfection

1. Cell culture: PC12 cells (clone 251; *5*) are cultured at 37 °C in 10% CO_2 at 90% relative humidity in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L glucose) supplemented with 10% horse serum, 5% fetal calf serum, 4 m*M* L-glutamine, 60 U/mL penicillin and 60 U/mL streptomycin (*see* **Note 1**).
2. Poly-L-lysine solution: 100 µg/mL poly-L-lysine (molecular weight ~ 300,000 kDa) in sterile double-distilled (dd) water. For storage, prepare a 20X stock solution, sterilize the solution by filtering through a 0.2-µm filter, and keep 2-mL aliquots at –20 °C.
3. Glass coverslips: Use round, 100-µm thick glass coverslips with a 25-mm diameter (*see* **Note 2**).
4. Cytomix: 120 m*M* KCl, 10 m*M* KH_2PO_4, 10 m*M* K_2HPO_4, 0.15 m*M* $CaCl_2$, 2 m*M* ethylene glycol-bis(2-amino-ethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 m*M* $MgCl_2$, 2 m*M* adenosine triphosphate (ATP), 5 m*M* glutathione, and

25 m*M* HEPES-KOH, pH 7.6 *(6)*, sterilized by filtering through a 0.2-μm filter. Store 1.5-mL aliquots at –20 °C.

5. NPY-GFP plasmid: The plasmid encodes a fusion protein of human neuropeptide Y (NPY) fused C-terminally to enhanced GFP (EGFP) in the mammalian expression vector pEGFP-N1 (Clontech) *(4)*. The concentration of the plasmid should not be lower than 0.5 μg/μL.

2.2 *Generation of Membrane Sheets*

1. Sonication buffer: 120 m*M* potassium glutamate, 20 m*M* potassium acetate, 10 m*M* EGTA, 4 m*M* $MgCl_2$, 2 m*M* ATP, 0.5 m*M* dithiothreitol, and 20 m*M* HEPES-KOH, pH 7.2. Store 50-mL aliquots at –20 °C.
2. Sonifier: A Branson sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) equipped with a 2.5-mm sonication tip can be used.
3. Sonication chamber: Use an approx 9-cm dish as the sonication chamber. The sonication tip of the sonifier is centered within the dish (Fig. 1) and its distance to the bottom of the dish is adjusted to 1 cm.

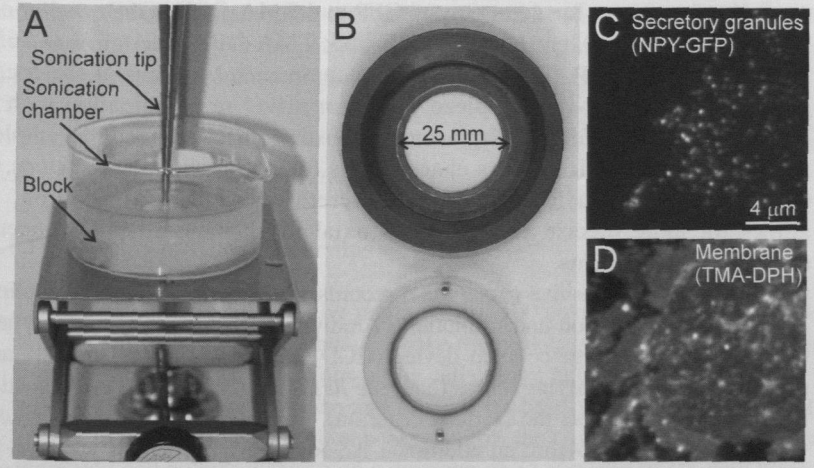

Fig. 1 Generation of membrane sheets with fluorescent docked secretory granules. **(A)** The sonication tip is centered in the sonication chamber at a distance of 1 cm between tip and bottom of the dish. To this end, a metal block 1 cm high can be put in the chamber and used as reference. Then, a glass cover slip is put below the tip (cell side up), and a sonication pulse is applied. The coverslip is taken out of the sonication chamber and mounted quickly in the microscopy chamber. **(B)** The microscopy chamber has to allow for quick mounting of the glass coverslip, for example, by sealing the chamber by screwing in an insert (we found a circular chamber to be easiest to handle, especially when the thread between chamber body and insert is large and the insert material is plastic). **(C)** Image of a membrane sheet in the green channel (visualizing NPY-GFP in secretory granules) and **(D)** the blue channel (visualizing phospholipid membranes stained by TMA-DPH).

2.3 Monitoring Exocytosis by Fluorescence Microscopy

1. Microscopy chamber: Use an open chamber that allows mounting of the glass coverslip within less than 1 min (Fig. 1).
2. Calcium-buffered solutions: For concentrations of free calcium in the range from 0 to 1.35 μ*M*, use solutions containing 10 m*M* EGTA and 1–9 m*M* $CaCl_2$ (*see* **Note 3**). The $[Ca^{2+}]_{free}$ is calculated from the K_d of EGTA for Ca^{2+}, which is dependent on ionic strength, temperature, and pH (for dissociation constants in 0.1 *M* KCl, *see Ref. 7*). For higher $[Ca^{2+}]_{free}$, use diethylenetriaminepentaacetic acid (DPTA) instead of EGTA (assuming a Ca^{2+} dissociation constant of 81 μ*M*; *8*).
3. TMA-DPH solution: For a saturated solution of TMA-DPH [1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene-*p*-toluensulfonate], add 10–20 mg TMA-DPH powder to 2 mL phosphate-buffered saline solution (137 m*M* NaCl, 2.7 m*M* KCl, and 8.1 m*M* Na_2HPO_4, pH 7.3). After vortexing, undissolved TMA-DPH is spun down, and the supernatant is used for experiments. For membrane staining, add 25 μL saturated TMA-DPH solution to 0.5 mL chamber buffer.
4. Fluorescence microscopy: A standard epifluorescence microscope with an 100 × magnifying objective lens is used (for an example, *see Ref. 4*). Use the following filters for detection of GFP and TMA-DPH: GFP, excitation BP 450–490, BS 510, emission BP 515–565; TMA-DPH, excitation G 365, BS 395, emission LP 420. It is highly recommended to control the focal position using a low-voltage piezo translator driver in combination with a linear variable transformer displacement sensor/controller (for an example, *see Ref. 3*). This device allows changing the focal plane in 100-nm steps, a prerequisite for focusing properly on secretory granules. In addition, it allows for more precise corrections of axial focal plane drifts that occur during the experiments.
5. Camera: A highly sensitive camera that records at diffraction-limited resolution is required for detection and resolution of individual secretory granules. When back-illuminated charge-coupled device (CCD) cameras with large pixels are used, additional magnifying lenses (e.g., *see Ref. 4*) are required to avoid spatial undersampling (to meet the Nyquist criterion). For instance, for camera pixels with a size of 24 × 24 μm, an additional 2.5 × magnifying lens is required to obtain 96-nm pixel sizes in the image.

2.4 Data Analysis

For image acquisition and data analysis, the Metamorph (Universal Imaging Corp., West Chester, PA) program can be used.

3 Methods

The optical readout of the assay requires fluorescent labeling of secretory organelles (*see* **Note 4**). For GFP labeling of secretory granules, we transfect a plasmid for expression of human NPY fused C-terminally to GFP (NPY-GFP) into PC12 cells via cell electroporation *(4)*. In principle, other cell types or secretory organelle markers can also be used (*see* **Notes 1 and 5**). Transfected cells are plated onto poly-L-lysine-coated glass coverslips and are used 2 d after electroporation (*see* **Note 6**).

For generation of membrane sheets, coverslips are treated with an ultrasound pulse that removes the upper parts of the cells and leaves behind the flat, glass-adhered basal plasma membrane with docked secretory granules (*see* **Note 7**). During generation and screening for a suitable membrane sheet, the preparation is kept in calcium-free solution to avoid triggering of exocytosis.

Once a membrane sheet has been selected and if necessary treated with biochemical reagents, the initial distribution and status of secretory granules are documented by taking an image. For triggering exocytosis, the buffer is exchanged with a solution containing a micromolar concentration of free calcium, and the recording of an image sequence is subsequently started. Exocytosis of single secretory granules is documented in the resulting image sequence, in which changes of granule intensities indicate a fusion event (*see* **Note 8**). After the experiment, the phospholipids of the membrane sheet are visualized by staining with TMA-DPH to document the integrity of the plasma membrane. Data are analyzed, and exocytosis is presented as a percentage of granules that underwent exocytosis.

3.1 Cell Culture and Transfection

3.1.1 Coating of Glass Coverslips

1. Glass coverslips are cleaned by putting about 100 coverslips in a 500-mL glass beaker containing 100 mL ethanol. Shake the beaker gently for 1–2 h. Continue working under sterile conditions in the cell culture hood.
2. Take out coverslips one by one and flame them briefly (take precautions not to set fire to the ethanol in the glass beaker), allow the coverslips to cool, and place them into cell culture dishes with a diameter of 35 mm (use of six-well plates is recommended). The rim of the coverslips should have no contact with the wall of the dish; otherwise, plated poly-L-lysine solution or cell suspension will not remain on the coverslips.
3. Pipet about 500 µL poly-L-lysine solution onto each coverslip. Make sure that the coverslip is evenly covered with the solution. After 20 min, suck off the poly-L-lysine solution and wash once briefly with about 2 mL sterile ddH_2O.
4. After 2 h of air drying, coverslips can be used for experiments or can be stored at 4 °C up to several weeks.

3.1.2 Cell Transfection

1. Grow PC12 cells in 75-cm^2 flasks. Passage them 1:2 when cells become almost confluent. Cells are detached from the substrate by trypsination, and the suspension is divided into two halves. Centrifuge the suspensions and use one pellet for the new passage and the other for transfection by electroporation. The cell pellet for passaging is resuspended and triturated in medium, and cells are transferred into a new flask.
2. The cell pellet for transfection is resuspended and triturated in 1.5 mL cytomix.
3. Transfer 350 µL of the cytomix cell suspension (should contain approx 2×10^6 cells) into an electroporation cuvette (2-mm gap between electrodes; total volume about 0.5 mL) and add 20–50 µg NPY-GFP plasmid deoxyribonucleic acid (DNA) (not more than 50 µL). Gently resuspend the cells (as they settle) and apply a 1150-V, 50-µF, and 50-Ω pulse using a cell electroporator. Transfer the cell suspension from the cuvette into a tube containing 3 mL medium.
4. Resuspend cells and plate 500 µL per coverslip. Make sure that cells are evenly distributed. Wait for 20 min to allow cells to settle and then carefully add 2 mL medium to each coverslip, pipeting the solution at the wall of the dish. Cells are transferred into the incubator and are ready to use for experiments after 2–3 d.

3.2 *Generation of Membrane Sheets*

1. Fill the sonication chamber with 200 mL ice-cold sonication buffer and keep it on ice.
2. Place a coverslip on the bottom of the chamber and center it. Transfer the chamber to the sonication device and position the tip of the sonication device above the glass coverslip at a distance of 1 cm (Fig. 1). Apply one 100-ms pulse at low energy (*see* **Note 9**).
3. Mount the coverslip in a microscopy chamber and fill it with sonication buffer containing reagents for biochemical treatment (e.g., ATP derivatives, rat brain cytosol, or SNARE [soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor] interacting proteins) of the plasma membrane if required (*see* **Note 10**). Start a timer (*see* **Note 11**).

3.3 *Monitoring Exocytosis by Fluorescence Microscopy*

1. Mount the microscopy chamber on the microscope and find a suitable membrane sheet for the experiment. Membrane sheets should be large with a high number of brightly fluorescent secretory granules (Fig. 1). This procedure takes between 5 and 10 min, setting a limitation between membrane sheet generation and stimulation of exocytosis. Use a fixed time window between membrane sheet generation and triggering of exocytosis (e.g., 10 min).
2. Once a membrane sheet has been found, wait for the chosen time window to elapse (*see* step 1) after mounting of the coverslip in the chamber. Image the

membrane sheet, add a buffer containing calcium-buffered solution, and start the image recording. For solution exchange, use a pipet. Using a back-illuminated CCD camera system, approx 100 images can be taken before GFP signals are lost because of bleaching. It is recommended to take not more than 50 images at a frequency that depends on the experimental time window (e.g., 2 images/min for 25 min, with exposure times of about 1 s).

3. After monitoring exocytosis, the studied membrane sheet should be visualized by a lipophilic dye to document the continuity of the plasma membrane. To this end, add TMA-DPH and image the preparation in the blue channel.

3.4 *Data Analysis*

1. For statistical reasons, analyze only membrane sheets with at least 15 secretory granules. Place circles on individual secretory granules visible in the first image (before buffer exchange).
2. Playing the image sequence, granules should show no lateral mobility. Regarding granule intensities, they might be stable, become dimmer, become brighter, or lose the signals. The last three types indicate exocytosis (*see* **Note 8**). For each granule, indicate the type of behavior, and if necessary the time-point when the exocytotic event occurred can be documented (*see* **Note 12**).
3. Express exocytotic activity as percentage of active secretory granules or plot events against time after normalizing to the total number of secretory granules on a membrane sheet (only applicable if a membrane sheet contains a sufficiently high number of granules and if time resolution of the experiment is low).
4. For each condition, approx 10 membrane sheets should be analyzed and averaged.

3.5 *Two Channel Recordings*

For recording exocytotic activity, only the green channel is required, and for documenting the integrity of the membrane sheets the blue channel is used. Using the red or far-red channels, simultaneous immunostainings *(4)*, fluid phase uptake during exocytosis followed by direct retrieval of granules *(3)*, or fluorescent-labeled bound protein can be monitored.

4 Notes

1. Other cell types can also be used; for example, pancreatic β-cell lines and bovine chromaffin cells have been tested. The only requirement is that cells attach firmly to the glass coverslip.

2. Shape and size of the coverslips do not matter as long as they are not thicker than 100 µm and larger than several square centimeters. This minimum area is recommended because the efficiency of membrane sheet generation is low, and screening a larger area for suitable membrane sheets is required. In general, round coverslips can be mounted quicker into a microscopy chamber than square coverslips.
3. Free-calcium concentrations in the range of 1–10 µ*M* have been reported to be optimal for triggering exocytosis from PC12 cells in in vitro systems. When setting up the assay, a range of $[Ca^{2+}]_{free}$ should be tested to find out the best condition. Please note that higher $[Ca^{2+}]_{free}$ can become inhibitory for exocytosis.
4. This can be achieved by incubating cells with the fluorescent dye acridine orange, which accumulates in acidic compartments like secretory granules (*9*), or by overexpression of a GFP-labeled peptide hormone that is sorted into secretory granules (2). Although cell transfection is necessary as an additional step for GFP labeling, it is the method of choice compared to acridine orange because it causes less photodamage, is more resistant to bleaching, and labels secretory organelles more specifically.
5. The granule marker should be releasable. If an integral secretory organelle protein is used, detection of exocytosis is only possible if GFP is localized inside the granule lumen and the lumen is acidic. In this case, exocytosis causes lighting up of the pH-sensitive GFP (e.g., *see Ref. 10*).
6. Expression and sorting of the protein takes 2 d, with maximal expression within the first day. After more than 4 d, GFP fluorescence is lost because expression has stopped, and the NPY-GFP produced has a turnover rate of about 24 h.
7. Secretory granules only fluoresce when the original cell was expressing NPY-GFP (the rate of transfection is between 10% and 30%), and often membrane sheets without secretory granules are generated. For this reason, a suitable membrane sheet has to be found by screening large areas on the glass coverslip.
8. Fusion results in granules becoming brighter (dequenching of the pH-sensitive GFP), dimmer (partial release of GFP), or losing their signal (emptying of granules).
9. The right energy setting of the sonication device has to be found out by testing the range of available settings. First, the pulse length is adjusted as short as possible (usually 100 ms), and then one pulse at lowest energy is applied to a coverslip. The cells adsorbed to the glass create slight turbidity. If after sonication the turbidity still covers the entire coverslip, the pulse energy is too low. Take another coverslip and increase the energy stepwise until sonication generates a clearing. The stronger the pulse, the larger is the clearing, finally leading to removal of all cells. The best setting is obtained if a large, starlike clearing is formed.
10. The buffer should contain EGTA to avoid triggering of exocytosis by traces of calcium prior to stimulation. In the absence of Mg-ATP, a loss of exocytotic activity within minutes is observed.

11. Treatments should not take longer than 10 min as run down of SNARE activity, for example, is observed after about 20 min.
12. Manual counting of exocytosis is recommended if only a low number of experimental conditions are tested; otherwise, algorithms for detection of exocytotic activity can be developed.

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