Video Article

Monitoring the Effect of Osmotic Stress on Secretory Vesicles and Exocytosis

Hoda Fathali[1], Johan Dunevall[1], Soodabeh Majdi[2], Ann-Sofie Cans[1]

[1]Department of Chemistry and Chemical Engineering, Chalmers University of Technology

[2]Department of Chemistry and Molecular Biology, University of Gothenburg

Correspondence to: Ann-Sofie Cans at cans@chalmers.se

URL: https://www.jove.com/video/56537
DOI: doi:10.3791/56537

Keywords: Neuroscience, Issue 132, Chromaffin cells, osmotic pressure, dense core vesicles, neurotransmitter concentration, quantal size, exocytosis, amperometry, intracellular cytometry, transmission electron microscopy

Date Published: 2/19/2018

Citation: Fathali, H., Dunevall, J., Majdi, S., Cans, A.S. Monitoring the Effect of Osmotic Stress on Secretory Vesicles and Exocytosis. *J. Vis. Exp.* (132), e56537, doi:10.3791/56537 (2018).

Abstract

Amperometry recording of cells subjected to osmotic shock show that secretory cells respond to this physical stress by reducing the exocytosis activity and the amount of neurotransmitter released from vesicles in single exocytosis events. It has been suggested that the reduction in neurotransmitters expelled is due to alterations in membrane biophysical properties when cells shrink in response to osmotic stress and with assumptions made that secretory vesicles in the cell cytoplasm are not affected by extracellular osmotic stress. Amperometry recording of exocytosis monitors what is released from cells the moment a vesicle fuses with the plasma membrane, but does not provide information on the vesicle content before the vesicle fusion is triggered. Therefore, by combining amperometry recording with other complementary analytical methods that are capable of characterizing the secretory vesicles before exocytosis at cells is triggered offers a broader overview for examining how secretory vesicles and the exocytosis process are affected by osmotic shock. We here describe how complementing amperometry recording with intracellular electrochemical cytometry and transmission electron microscopy (TEM) imaging can be used to characterize alterations in secretory vesicles size and neurotransmitter content at chromaffin cells in relation to exocytosis activity before and after exposure to osmotic stress. By linking the quantitative information gained from experiments using all three analytical methods, conclusions were previously made that secretory vesicles respond to extracellular osmotic stress by shrinking in size and reducing the vesicle quantal size to maintain a constant vesicle neurotransmitter concentration. Hence, this gives some clarification regarding why vesicles, in response to osmotic stress, reduce the amount neurotransmitters released during exocytosis release. The amperometric recordings here indicate this is a reversible process and that vesicle after osmotic shock are refilled with neurotransmitters when placed cells are reverted into an isotonic environment.

Video Link

The video component of this article can be found at https://www.jove.com/video/56537/

Introduction

Chromaffin cells in adrenal glands are neuroendocrine cells that release neurotransmitter molecules into the blood stream. This occurs through a cellular process that involves the docking and fusion of neurotransmitter-filled vesicles, resulting in content release from vesicles to the extracellular space in a process called exocytosis. The neurotransmitters (adrenaline and noradrenaline) in chromaffin cells are actively transported by membrane proteins into large dense core vesicles (LDCVs) and stored at high concentrations (~0.5-1 M)[1,2]. Accumulation of neurotransmitters inside the LDCVs is accomplished by the affinity of catecholamine molecules to the intravesicular dense core protein matrix comprised of chromogranin proteins (~169 mg/mL)[3,4,5,6], and an intravesicular cocktail solution containing key components for catecholamine loading and storage into the vesicle such as ATP (125-300 mM)[7], Ca^{2+} (50-100 µM in the solution and ~40 mM bound to the protein matrix)[8], Mg^{2+} (5 mM)[9], ascorbate (10-30 mM)[10], and a pH of ~5.5[11,12]. The LDCVs maintain an iso-osmotic condition with the cell cytoplasm (310 mOsm/kg)[13], even though the theoretical solute concentration inside the vesicles sum up to more than 750 mM. The composition of the intravesicular components is not only essential for the loading and storage of catecholamines, but also for aggregation of solutes to the dense core protein matrix. This significantly reduces the osmolarity of the vesicles is significantly reduced and can affect the amount catecholamine that is released during exocytosis[5,6].

Studies on the effect of extracellular osmotic pressure on the exocytosis process by amperometric recording have reported that high extracellular osmotic pressure inhibits exocytosis activity and reduces the number of neurotransmitters secreted from single vesicle compartments[4,14,15,16,17,18,19]. The explanations of these observations have speculated on the possible enhancement of macromolecular crowding in the cell cytoplasm inhibiting vesicle fusion events, and an alteration in membrane biophysical properties affecting the number of neurotransmitters released during exocytosis. These thoughts assumed that high extracellular osmotic stress does not affect the vesicle quantal size, which defines the number of neurotransmitter molecules stored in a vesicle compartment at prior stage of triggered exocytosis[14,15,17,19,20,21]. In amperometric measurements of exocytosis release in single cells, a carbon fiber disc microelectrode is placed in close contact with the cell surface, creating an experimental set up mimicking the synapse configuration, where the amperometric electrode serves as a postsynaptic detector (**Figure 1**)[22,23]. By stimulating a cell to exocytosis, one can induce neurotransmitter-filled vesicles to fuse with the cell plasma membrane and release part or the full vesicle content into the extracellular space. These neurotransmitter molecules released at the surface of the electrode can be detected electrochemically if the neurotransmitters are electroactive (*e.g.*, catecholamines) by applying a redox potential of +700 mV vs

Copyright © 2018 Journal of Visualized Experiments

a Ag/AgCl reference electrode. Consequently, a series of current spikes mark the detection of individual exocytosis events. From the current versus time trace in an amperometric recording, the area under each single amperometric spike represents the charge detected per exocytosis event and can be converted to the mole of neurotransmitters released, using the Faraday equation. Hence, the amperometric recordings provide quantitative information on the amount neurotransmitters expelled from single exocytosis events and report on the frequency of exocytosis events, but do not present quantitative information on the secretory vesicles content before vesicle fusion and neurotransmitter release has been initiated.

Therefore, to get a better understanding of how secretory vesicles in the cell cytoplasm respond to extracellular osmotic stress before the cell is triggered to undergo exocytosis, other complementary analytical methods can be used to enrich this information. For instance, to investigate if osmotic stress alters vesicle volume, transmission electron microscopy (TEM) imaging analysis can be used to measure the vesicle size of cells after chemical fixation. To examine if osmotic stress affects vesicle quantal size, a recently developed amperometric technique called intracellular electrochemical cytometry, can be applied for quantification of vesicle neurotransmitter content at secretory vesicles in their native state when still residing in the cytoplasm of live cells[26]. In the intracellular electrochemical cytometry technique, a nanotip cylindrical carbon fiber electrode is gently inserted into the cytoplasm of live cells and, by applying a +700 mV potential to this electrode (vs a Ag/AgCl reference electrode), the catecholamine content in vesicles can be quantified by the detection of a redox current spike from single vesicles colliding, adsorbing, and subsequently stochastically rupturing at the electrode surface and thereby releasing their contents against the electrode surface[26]. Hence, in the amperometric current versus time trace, each single vesicle rupturing event can result in a current transient and, by integrating the area of each current spike, the vesicle quantal size can be calculated using Faraday´s law.

Therefore, by linking the quantitative information gained from vesicle size measurements using TEM imaging together with vesicle quantal size analysis, as recorded by intracellular electrochemical cytometry, vesicle neurotransmitter concentration can also be determined. This allows vesicle characterization when cells are exposed to different osmotic conditions and provides a better view on how vesicles may respond to extracellular osmotic stress at the stage prior to exocytosis. The results from combining these methods have shown that in the presence of extracellular high osmotic pressure, vesicles shrink and adjust their quantal size and comparing the quantitative information on the relative changes from these measurements shows that while shrinking, vesicles adjust their contents and size to maintain a constant neurotransmitter concentration[24]. Thus, this understanding is valuable in connecting to the observations made on neurotransmitter release in cells exposed to osmotic stress. In these protocols, we describe the use of these three complementary methodologies that allow the characterization of how secretory vesicles in their native environment respond to extracellular osmolality and the effects of this response on the exocytosis process. In addition to our previous observations regarding the effect of high osmotic pressure on exocytosis[24], we present additional experiments that describe cell recovery after osmotic shock and the effect of multiple barium stimulations in chromaffin cells.

Protocol

1. Cell Culture of Bovine Chromaffin Cells Isolated by Enzymatic Digestion from Adrenal Glands

1. To isolate chromaffin cells collected from bovine adrenal glands, sterilize cells with 70% ethanol solution. Trim away fat and connective tissue using a scalpel. To remove blood, rinse the adrenal veins using Locke's solution (NaCl 154 mM, KCl 5.6 mM, NaHCO3 3.6 mM, hydroxyethyl piperazineethanesulfonic acid (HEPES) 5.6 mM at pH 7.4) that has been thermostated to 37 °C.
2. To digest the tissue, inflate each gland by injecting approximately 2.5 mL of warm sterile-filtered 0.2% collagenase P solution through the adrenal vein using a syringe and incubate the tissue for 20 min at 37 °C. Examine the glands to ensure the digestion process of the medulla is completed. The tissue should feel soft and not tense.
3. Collect the digested medulla by snipping off the glands in the longitudinal direction. Mince the medulla tissue using a scalpel. Filter the tissue suspension over a steel sieve and dilute with Locke's solution to approximately a 50 mL volume to reduce the activity of collagenase P.
4. Pellet the cells at 300 x g in a centrifuge for 10 min at room temperature and collect into 50 mL sterile test tubes. Re-suspend the obtained pellet in 20 mL Locke's solution and filter the solution over a sterile 100-µm nylon mesh into tubes.
5. Mix the chromaffin cell suspension with sterile Percoll (1:1) and centrifuge the cell solution at 18,600 x g for 20 min at room temperature. Collect the top layer of the density gradient and filter the solution over a 100-µm nylon mesh into tubes.
6. To exclude Percoll, dilute the cell suspension with Locke's solution and centrifuge at 300 x g for 10 min at room temperature before re-suspending the pellet in Locke's solution. The estimated cell density of the isolated cell suspension is approximately 4 million cells/mL.
7. For amperometric recording of exocytosis and intracellular cytometry measurements, plate chromaffin cells in collagen IV coated 60 mm plastic dishes at a density of about 17.5 × 10^3 cells/cm^2 and incubate the cells at 37 °C in a 5% CO_2 environment. Perform the electrochemical experiments within 1-3 days of cell culture.
8. For the TEM imaging experiments, plate chromaffin cells in 75 cm^2 cell culture flasks at a density of 7-8 million cells per flask and incubate at 37 °C in a 5% CO_2 environment for 1 day.

2. Single Cell Exocytosis Amperometry Experiments[24]

1. To fabricate carbon fiber microelectrodes for these experiments, take a glass capillary with an outer diameter suitable for the electrode holder at the head stage that will be used in these experiments. Use a borosilicate glass capillary with an outer diameter of 1.2 mm and an inner diameter of 0.69 mm.
    1. Connect one of the capillary ends to a water aspiration tube. Place 5 µm diameter carbon fibers on something, such as a piece of white paper, to enhance visualization.
    2. Identify a single carbon fiber and hold the fiber down on one end with a finger to keep the carbon fiber in place while positioning the open end of the capillary in proximity to the free end of the carbon fiber. Gently aspirate the carbon fiber into the glass capillary so that the carbon fiber sticks out through both ends of the capillary. Move away the aspiration force.

2. Place the capillary with the carbon fiber into a micropipette puller and pull the glass capillary into two separate glass pipette tips. To disconnect the carbon fiber that is connected between the two glass tips, use a pair of scissors to cut the carbon fiber and gain two carbon fiber microelectrodes.
3. Under a microscope, place the carbon fiber on top of a thicker microscope slide that allows manual cutting of the carbon fiber at the edge of where the fiber is extending from the glass capillary coating using a scalpel.
4. After cutting the carbon fiber, leaving a glass coated carbon fiber tip, insert a few mm of the electrode tip into an Epoxy solution for 10 min to pull up epoxy and seal any possible open space between the carbon fiber and the surrounding glass. Lift the electrodes slowly out of the epoxy solution to prevent bulky glue drops from forming at the tip of the electrode.
5. Place the electrodes on a holder (*e.g.*, a wooden flat stick) with two-side sticky heat resistant tape to which electrodes can be attached. Bake the epoxy treated electrodes overnight in an oven at 100 °C. The electrode tips easily break if they come into contact with a surface, so ensure the electrodes always are safely stored using a holder where the electrodes are fixed in place and tips do not risk being touched.
6. To obtain a flat disc electrode surface, place the electrode in the holder of a microgrinder and bevel each carbon fiber electrode at an angel of 45°. After beveling, mark the capillary on its topside using a permanent marker to later know how to locate the disc electrode surface at a 45° angle when placing the electrode near cells for the exocytosis measurement.
   NOTE: This step is important as in these experiments, the oval disc electrode needs to be placed flat on top of cells with its surface parallel to the surface of the Petri dish. Also, beveling electrodes are done the same day as experiments to ensure a fresh and clean electrode surface.
7. Before use, place each carbon fiber microelectrode in a test solution (*e.g.*, 0.1 mM dopamine in PBS buffer (pH 7.4)) to monitor the steady state current using cyclic voltammetry. For a cyclic voltammetry scan, apply a triangle potential waveform from -0.2 V to +0.8 V vs a Ag/AgCl reference electrode at 100 mV/s to ensure good reaction kinetics data are obtained that are in agreement with theoretically calculated values for a 5 µm diameter disc carbon fiber microelectrode[25].
8. For amperometry recording of exocytosis, place the Petri dish with cultured chromaffin cells onto an inverted microscope. It is important to shield the microscope set up with a Faraday cage to eliminate electronic noise during the amperometry recording, due to the very small currents being measured. Use a microscope heating stage to maintain a temperature of 37 °C during the cell experiments .
9. Use a low-noise patch clamp instrument to apply a constant potential of +700 mV at the working electrode versus a Ag/AgCl reference electrode that is also placed in the Petri dish away from the working electrode. For recording exocytosis of chromaffin cells, digitalize the signal at 10 kHz and apply an internal low pass Bessel filter at 2 kHz to filter the recorded signal.
10. To perform amperometric recording at single cells, mount the freshly beveled and tested carbon fiber disc microelectrode in the electrode holder of the head stage that is used with the potentiostat.
    1. Gently place the electrode with the flat oval disc shape electrode surface facing down towards the apical surface of the cell and place the electrode in contact with the cell membrane using a micromanipulator.
    2. Adjust the distance from the electrode to the cell by monitoring the cell deformation caused by the electrode upon placement on top of the cell membrane and then carefully retract the electrode to a distance where the cell regains a shape close to its original cell shape. The ideal for kinetic and quantitative recording is to create a thin liquid film of a hundred nanometers to separate the electrode and cell surface, which are similar conditions for postsynaptic detection of chemical release in a synapse.

11. To stimulate the cells to exocytosis, position a glass micropipette with a tip size of 2-3 µm diameter, filled with 5 mM $BaCl_2$ solution at a distance of at least 20 µm away from the cell, to prevent any solution leaking from the pipette tip to influence the experiment and apply a 5 s injection pulse of 5 mM $BaCl_2$ solution at the cell surface to stimulate the cell to exocytosis.
12. To study the reversible effects of osmotic pressure on the exocytosis process, prepare an isotonic buffer solution (150 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 5 mM glucose, 10 mM HEPES, pH 7.4) with 310 mOsm/kg iso-osmotic pressure and a hypertonic buffer made by adjusting the NaCl concentration of the isotonic buffer solution with an osmolality corresponding to 730 mOsm/kg.
13. Place the cells in the isotonic buffer and stimulate the cell to exocytosis by applying a barium injection pulse while recording the amperometric current transients during the initiated exocytosis activity for approximately 3 min.
    1. To compare the exocytosis responses in isotonic conditions to hypertonic conditions, incubate the cells for 10 min in hypertonic buffer solution and thereafter apply a barium injection pulse to stimulate exocytosis and perform 3 min of amperometric recording.
    2. For the reversible response of cells, incubate cells again for 10 min in isotonic buffer solution and stimulate cells by applying a 5s barium injection pulse and perform 3 min of recording the exocytosis response from the cell.

14. As control experiments to determine the effect on exocytosis activity by multiple barium stimulations, perform an amperometric recording of exocytosis release from three consecutive $BaCl_2$ stimulations on the same cell placed in isotonic buffer and using the same time protocol for the consecutive cell incubation experiments with different osmolarity.

3. Intracellular Electrochemical Cytometry[24,26]

1. To fabricate electrodes for vesicle quantal size measurements, use the same materials and start preparing a 5 µm in diameter carbon fiber electrode according to the descriptions in sections 2.1 and 2.2 of microelectrode fabrication for amperometric exocytosis measurements.
2. Under a microscope, use a scalpel to cut the carbon fiber extending out of the glass tip so that a carbon fiber with a length ranging from 30 to 100 µm is left sticking out from the glass tip.
3. To prepare a flame etched tip of the carbon fiber electrode, use a butane flame. To achieve an evenly etched, cylindrical shaped electrode tip, hold the cylindrical shaped carbon fiber electrode while rotating it and place the carbon fiber extending from the glass into the blue edge of the butane flame until the tip of the carbon develops a red color. This often takes less than 2 s. If successful, this results in an etched electrode tip size of 50-100 nm in diameter (a SEM image of such tip is shown in **Figure 5B**). After flame etching, place the electrode under a microscope to evaluate the electrode tip.
4. Insert the cylindrical nanotip microelectrode in epoxy solution for 3 min followed by a 15 s dipping of the electrode tip into an acetone solution. This allows epoxy to seal the potential gap space between the carbon fiber and the insulating glass capillary wall, while the acetone clears the epoxy off the etched carbon fiber electrode surface. To cure the epoxy, bake the electrodes in an oven overnight at 100 °C.
5. Before use, test the steady state current of each carbon fiber microelectrode, as explained in section 2.7 using cyclic voltammetry. For experiments, only use electrodes that display a plateau current around 1.5-2.5 nA[27].

6. For intercellular amperometry measurements, place the cells at the microscope as described in 2.8 and use the same experimental settings of the potentiostat as described in 2.9.
7. To perform intracellular amperometric measurement, and to prevent significant physical damage to the cell, insert the nanotip cylindrical microelectrode into the cell by adding a gentle mechanical force, just enough to push the electrode through the cell plasma membrane and into the cell cytoplasm using a micromanipulator.
8. After insertion, and with the cell membrane sealed around the cylindrical electrode, start the amperometric recording in situ at the live cell. At the oxidation potential that is applied to the electrode, vesicles will adsorb to the electrode surface and stochastically rupture. Hence, there is no need for any kind of stimulus to initiate this process.
9. To determine the osmotic effect on vesicle quantal size, collect the intracellular cytometry measurements from a group of cells that have been incubated in isotonic and in hypertonic buffer using the experimental condition as described in section 2.13.

4. Data Analysis of Amperometry Recordings

1. To analyze the recorded amperometry data from exocytosis and intracellular vesicle quantal size analysis, use a software program that allows analysis of current transients in the recorded current versus time trace so that kinetic peak parameters and the integrated total charge for individual current peaks can be determined. For data analysis, we have used software developed in the Sulzer lab that was written for the data analysis program Igor[28].
2. When analyzing the amperometric spikes, select a threshold limit for peaks of three times the root-mean square (RMS) standard deviation of the noise for each recording.
3. Collect the amperometric spikes from each cell recording manually to prevent false spikes that do not follow the Gaussian shape, double spikes which can be the result of merged exocytosis events, and only accept Gaussian shaped spikes with a threshold of three times higher than the RMS noise.
4. Collect the data for total charge per single amperometric peak and use Faradays law (N=QnF) to calculate the molar amount of catecholamine neurotransmitter detected per exocytosis or intracellular single vesicle rupture event, where N is the moles of neurotransmitters going through a redox reaction at the electrode surface, Q=the total charge detected under each spike, n=number of electrons transferred in the redox reaction (n=2 for catecholamines), and the Faraday´s constant F=96485 C/mol.
5. Perform statistical analysis of the data collected by first calculating the average charge detected per event for each cell. Then for variance between cells, calculate the average charge between cells and use a student's t-test between cells assuming unequal variance. This study used the MATLAB program for statistical analysis.

5. TEM Imaging for Vesicle Size Analysis

1. To perform TEM imaging of cells at the different osmotic conditions, first incubate the cells in isotonic or hypertonic buffer for 10 min at 37 °C in a 5% CO_2 environment before chemical fixation of cells.
2. Perform cell fixation using the Karnovsky fixative method[29]. Using this method, incubate the cells with a solution containing 0.01% sodium azide, 1% formaldehyde, and 1.25% glutaraldehyde in which the sample can be stored at 4 °C.
3. After fixation, wash the cells with 0.15 M sodium cacodylate buffer.
4. To stain the cell samples post fixation, and in preparation for TEM imaging, incubate the cells with a 1% osmium tetroxide solution for 2 h at 4 °C, followed by 1 h incubation in 0.5% uranyl acetate solution at room temperature in the dark.
5. In a final fixation step, dehydrate the cells first by rinsing the cells in 100% ethanol and then rinse cells with acetone.
6. Embed the cell samples in epoxyresin and thereafter perform centrifugation at 4,000 x g for 30-40 min and allow the cell sample to polymerize at 40 °C for 15 h followed by 48 h incubation at 60 °C.
7. In preparation for imaging, section the cell sample embedded in Agar 100 resin into slices with thickness of 60 nm using an ultramicrotome.
8. Subject the cells to a 4% uranyl acetate/25% ethanol solution for 4 min followed by 20 s of rinsing with water. Wash the cells with Reynolds lead citrate for 3 min, followed by 20 s of rinsing with water.
9. Perform transmission electron microscopy imaging of cell samples. In our experiments, we have used a transmission electron microscope that has been operated at 120 kV.
10. From each recorded image of a cell section illustrating the cell ultrastructure that displays a population of the vesicles in the cell cytoplasm, first calibrate the image pixel size by relating pixels to the recorded scale bar in each TEM image. Use imaging software to determine the vesicle sizes at each image of cells exposed to isotonic or hypertonic conditions. In our experiments, we used the software ImageJ for image analysis. It is worth noting that for image analysis of cellular ultrastructure from TEM images of cells, size adjustment of these measurements based on thickness of cell sections needs to be considered and have been previously described by Almers´s lab[30].

Representative Results

We here describe the protocol for how combining TEM imaging together with two electrochemical methodologies, carbon fiber amperometry and intracellular electrochemical cytometry, can provide information that gains a broader view alluding to the effect of extracellular osmotic pressure on secretory vesicles and the exocytosis process. By comparing representative amperometric recordings of exocytosis release at single chromaffin cells using the experimental set up (shown in **Figure 1**), a significant reduction in exocytotic activity was displayed when cells were exposed to osmotic stress as compared to cells in isotonic conditions (**Figure 2A**)[24]. From these recordings and by using Faraday´s law, the total charge detected by each individual amperometric current spike was used to calculate the number of molecules expelled from single vesicle exocytosis events at cells exposed to the different osmotic conditions. By comparison, as displayed in **Figure 2B** by the smaller area of the average amperometric current spike detected by cells in hypertonic solution, fewer neurotransmitter molecules were released from each vesicle by cells sensing osmotic stress[24].

To determine the reversibility of this decline in the number of neurotransmitter molecules released, we performed amperometric recordings of exocytosis at cells exposed to a hypertonic environment and, subsequently, at cells after placed back into an isotonic environment. In these experiments, chromaffin cells were stimulated three consecutive times with $BaCl_2$ solution: first in an isotonic buffer, followed by a second

stimulation after cells were incubated for 10 min in a hypertonic solution and, finally, a third stimulation after 10 min cell incubation in isotonic conditions. The results as displayed in **Figure 3A** present that the quantity of neurotransmitters released was reduced by ~50% when cells were exposed to the hypertonic condition compared to the first Ba^{2+} stimulation in the isotonic condition. Subsequently, when cells were brought back to an isotonic environment and subjected to a third Ba^{2+} stimulation, the amount neurotransmitter released per exocytosis event was reversed back to the original amount recorded at the first stimulation, which confirmed previous observations[14]. Control experiments with three successive Ba^{2+} stimulations of cells in the isotonic condition (see **Figure 3B**) show that multiple sequential Ba^{2+} stimulations in isotonic conditions did not alter the amount neurotransmitter released during exocytosis. This suggests that vesicle quantal size is quickly and reversibly adjusted with extracellular osmolarity.

However, when analyzing the amperometric traces from these experiments in terms of exocytosis activity, it became evident, as displayed in **Figure 4A**, that exocytosis activity was significantly hampered when cells were exposed to hypertonic stress. During osmotic stress, exocytosis events were reduced to 12% of the activity at cells in isotonic condition. Subsequently, after the osmotic shock and cells were placed back into an isosmotic environment, cells regained 41% of their original exocytosis activity. Interestingly, the control experiments performed in isotonic conditions showed, as displayed in **Figure 4B**, that after performing three consecutive BaCl2 stimulations, the frequency of exocytosis events was reduced to 53% after a second stimulation and further down to 26% by the third stimulation compared to the first stimulation. Hence, it is clear that consecutive Ba^{2+} stimulations do not seem to affect the number of neurotransmitters released from each vesicle when exocytosis is triggered, but is significantly influencing the efficiency of the vesicle release process.

To investigate how secretory vesicles in their native environment are affected by extracellular osmotic stress in terms of vesicle volume or quantal size, vesicle size analysis using TEM imaging was combined with intracellular electrochemical cytometry at cells exposed to isotonic and hypertonic conditions. In the intracellular electrochemical cytometry experiments, a carbon fiber nanotip electrode was inserted into the cytoplasm of live chromaffin cells when placed in isotonic and hypertonic solution (as shown in **Figure 5**.) The resulting amperometric current spike was monitored of each vesicle in the cell cytoplasm colliding, adsorbing, and stochastically rupturing and releasing the vesicle contents at the surface of the amperometric electrode upon bursting[26]. The integrated total charge detected for each peak in the current versus time trace was used to calculate the average vesicle quantal size in each cell recording by Faraday´s law. These intracellular electrochemical cytometry measurements, shown in **Figure 6** and **Figure 7B**, demonstrated that vesicle quantal size at cells exposed to osmotic stress were significantly reduced compared to at cells in isotonic conditions. Comparing the magnitude of alteration in vesicle quantal size as measured by intracellular electrochemical cytometry, to the fractional decline in the quantity of neurotransmitter released during exocytosis at cells experiencing extracellular osmotic stress, showed a relative decrease of 60% in both quantal size and the amount neurotransmitter released compared to at cells in isotonic conditions (**Figure 7**)[24]. To relate the adjustment in vesicle quantal size to a potential variation in vesicle neurotransmitter concentration of cells experiencing osmotic pressure, TEM image analysis was performed to determine the vesicle size of cells exposed to isotonic and hypertonic conditions. Furthermore, the dark staining of the dense core protein matrix inside the LDCVs that is visualized in the TEM images as a dark sphere in the membrane bound vesicles was used to measure the volume of dense core matrix in these vesicles. As presented in **Figure 8**, vesicle size was reduced to 60% at cells exposed to extracellular osmotic stress compared to at cells in isotonic conditions. From the calculated volume of the measured diameter of the LDCV and the dense core, the volume of the surrounding halo solution that appears as a clear solution inside the LDCVs in the TEM images was also calculated. The summarized results from the TEM image analysis showed, as displayed in **Figure 8**, that during extracellular osmotic shock it is mainly the volume of the halo solution in the LDCVs that is reduced[24].

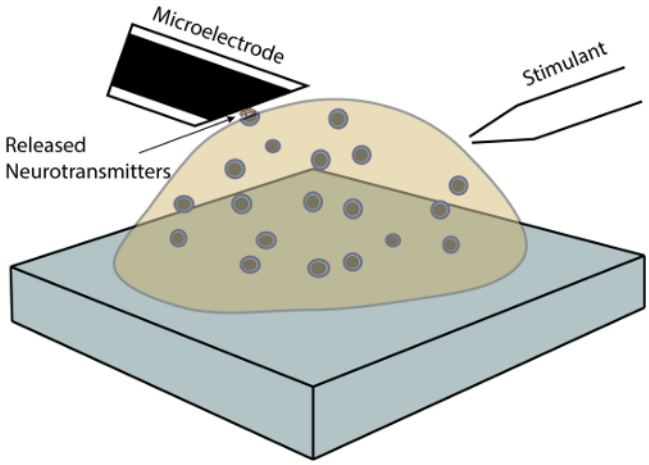

Figure 1: Single cell exocytosis amperometry. A schematic of the experimental set up for amperometric measurement of exocytosis at single chromaffin cells[24]. Please click here to view a larger version of this figure.

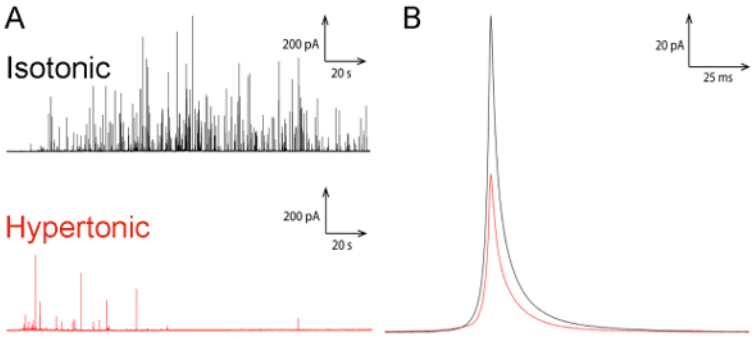

Figure 2: Amperometric traces from exocytosis measurements. (A) A representative amperometric recording of exocytosis at chromaffin cells in isotonic (black color) and in hypertonic (red color) extracellular environments. **(B)** Enlargement of an average amperometric spike from exocytosis measurement of chromaffin cells in isotonic (black) and hypertonic (red) extracellular environments[24]. Please click here to view a larger version of this figure.

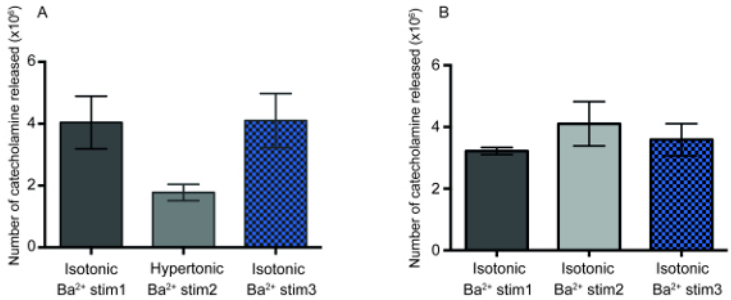

Figure 3: The reversibility of the number of catecholamines released at exocytosis after osmotic shock. (A) The number of molecules released during exocytosis from chromaffin cells (n=4) by three consecutive Ba^{2+} stimulations, with the first stimulation in isotonic, the second in hypertonic, and the third in isotonic buffer. The statistical results of the unpaired t-test are shown. The *p*-value for comparison of the first Ba^{2+} stimulation (Ba^{2+} stim 1) in isotonic buffer with the second Ba^{2+} stimulation (Ba^{2+} stim 2) in hypertonic buffer is *p*=0.1088 and the *p*-value for the comparison of the second Ba^{2+} stimulation in hypertonic solution with the third Ba^{2+} stimulation (Ba^{2+} stim 3) in isotonic buffer is p=0.059. **(B)** Control experiment demonstrating the amount of neurotransmitter molecules released at three consecutive Ba^{2+} stimulations of chromaffin cells (n= 4) in isotonic buffer. Please click here to view a larger version of this figure.

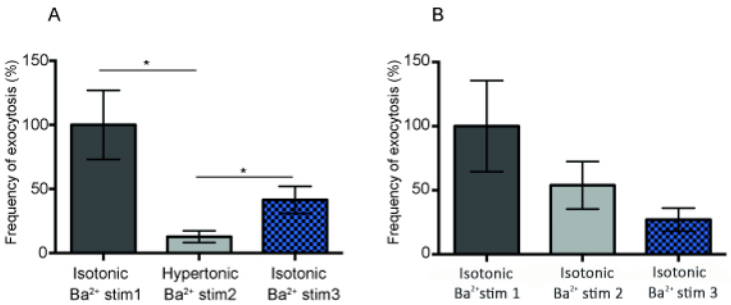

Figure 4: The effect of osmotic pressure on exocytosis activity. (A) The effect of extracellular osmolarity on exocytosis activity is presented as the frequency of exocytosis events when chromaffin cells (n= 4) are stimulated with barium solution in isotonic, then hypertonic, and finally in isotonic conditions. The values are presented as the average number of spikes from each cell and the average from all cells sampled (standard error of the mean (SEM)). The statistical significance of changes is presented using a t-test for unpaired data (the *p*-value for isotonic Ba^{2+} stimulation 1 and hypertonic Ba^{2+} stimulation 2 is *p*=0.0126 and the *p*-value for comparison of hypertonic Ba^{2+} stimulation 2 and isotonic Ba^{2+} stimulation 3 is *p*=0.037).***(B)*** Control experiment presenting the frequency of exocytosis events after three consecutive barium stimulations at chromaffin cells (n=4) in isotonic conditions. Please click here to view a larger version of this figure.

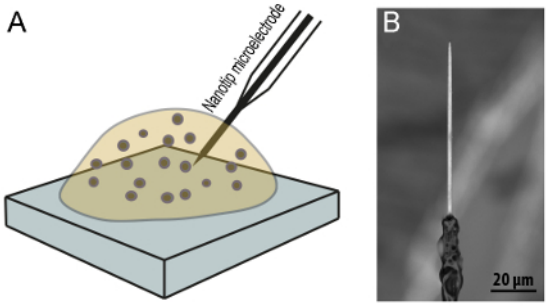

Figure 5: Intracellular vesicle electrochemical cytometry to monitor changes in vesicle quantal size. (A) A schematic of the experimental set up used for intracellular electrochemical cytometry. **(B)** A scanning electron microscopy image of a typical nanotip conical carbon fiber electrode[24]. Please click here to view a larger version of this figure.

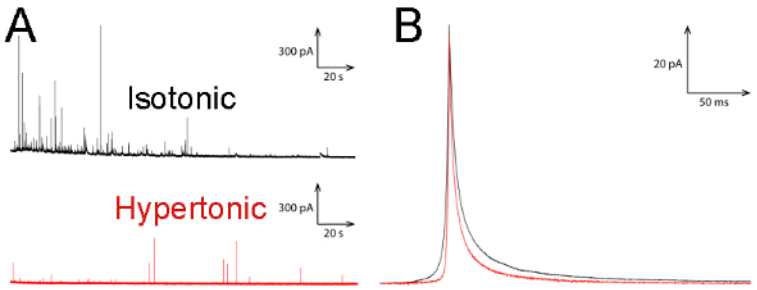

Figure 6: Intracellular electrochemical cytometry measurements showing (A) Representative traces of intracellular amperometric cytometry recordings at chromaffin cells in isotonic (black) and hypertonic (red) extracellular environments. **(B)** Enlargement of an average amperometric spike from intracellular cytometry measurement at chromaffin cells in isotonic (black) and hypertonic (red) extracellular environments[24]. Please click here to view a larger version of this figure.

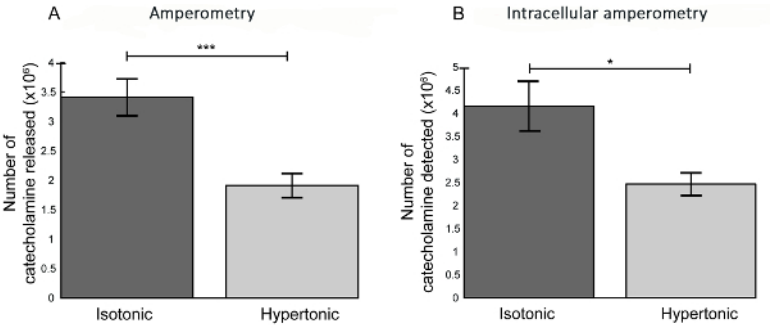

Figure 7: Quantification of the number of catecholamines released at exocytosis and determination of vesicle quantal size. (A) The average number of molecules released during exocytosis recordings at chromaffin cells in isotonic (n=22) and hypertonic (n= 20) environments. The values are presented as an average number of molecules released per exocytosis event from each cell and averaged from the cells sampled (± *SEM*). The statistical significance of changes are presented using t-test for unpaired data (*p*-value=0.0003) **(B)** The average number of molecules per vesicle as detected by intracellular electrochemical cytometry at chromaffin cells in isotonic (n=19) and hypertonic (n=16) conditions. The values are presented as the average number of molecules per spike as detected from each cell and averaged from all cells sampled (± *SEM*). The statistical significance of changes is presented using t-test for unpaired data (*p*- value=0.0108)[24]. Please click here to view a larger version of this figure.

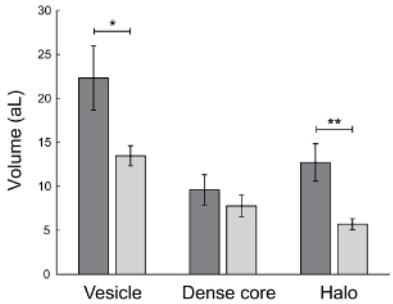

Figure 8: Effect of osmotic pressure on large dense core vesicle size. The calculated volume of the LDCVs, the dense core protein and the halo solution surrounding the dense core protein matrix was calculated in attolitres (aL) from image analysis of TEM images of chromaffin cells in isotonic (n=12) and hypertonic (n=9) buffers. The results were collected from an average of 311 vesicles per cell and averages from single cells (± *SEM*). The *p*-values are reported from unpaired t-tests comparing isotonic and hypertonic buffers. The *P*-value is 0.0385 (*) for vesicle volume, 0.3967 for dense core volume, 0.0047 (**) for halo volume[24]. Please click here to view a larger version of this figure.

Discussion

We here present a protocol and the advantages of combining three complementary analytical methods to analyze secretory vesicles and the exocytosis process to gain a better understanding of how a physical force such as osmotic pressure can affect secretory vesicles and the exocytosis process in secretory cells. These methods include carbon fiber microelectrode amperometry, which is an established method for recording exocytosis activity, intracellular electrochemical cytometry, which is used to determine quantal size of vesicles in their native environment, and transmission electron microscopy image analysis to monitor secretory vesicle volume. In this work, by collecting quantitative data from amperometry recordings of single vesicle exocytosis events at chromaffin cells exposed to an isotonic and a hypertonic extracellular environment, we confirmed that osmotic shock significantly reduces the amount neurotransmitters released during exocytosis and that these cells can reversibly recover and release the original quantity, if placed back into an isotonic environment (**Figure 3**).

The amperometric recordings provided information of the frequency of exocytosis events versus time and therefore can also be used to monitor changes in exocytosis activity at cells in different osmotic environments. This data can be interpreted and used to discern whether alterations in activity occur instantly or as a function of time. As we showed in previous work, although osmotic stress hampered exocytotic activity, it did not seem to hinder the fusion of the readily releasable pool of vesicles[24]. The exocytosis activity data can also be used to analyze the total exocytotic activity during a period of recording time. Here we present the accumulated number of exocytosis events from a 3-minute recording of exocytosis at chromaffin cells exposed to the two different osmotic conditions. These data show an inhibition in exocytosis activity of cells experiencing osmotic stress and that a partial recovery of this activity can be achieved after cells return to an extracellular isotonic environment. However, very importantly, the control experiments showed that multiple Ba^{2+} stimulations within the time frame used in these experiments caused a significant reduction in exocytosis activity by each time a cell was stimulated to secretion. By the third consecutive Ba^{2+} stimulation, only a third of the exocytosis activity was maintained, and clearly the barium, and perhaps also the timing of stimulations in these experiments, was affecting the vesicle cycle. This might be relevant for explaining why exocytotic activity was recovered in cells placed in isotonic solution after the osmotic shock and why these cells displayed a similar relative reduction in activity compared with cells in isotonic conditions after three sequential Ba^{2+} stimulations. Hence, amperometry recording of exocytosis provides information on secretory vesicles from the moment the vesicles are triggered to fuse with the plasma membrane, releasing neurotransmitters through the fusion pore. Thereby quantitative data on single vesicle neurotransmitter release and information on the exocytosis activity can be collected.

The quantity of neurotransmitter released can be regulated by the mode of exocytosis that is triggered, where either the full vesicle content or part of the content is released. By solely studying exocytosis release using carbon fiber amperometry that only detects what is expelled from a vesicle, it is difficult to distinguish if a change in the detected amount of neurotransmitter released is related to an alteration in the triggered mode of exocytosis, a change in the cellular biophysical properties affecting vesicle content release, or to changes in vesicle quantal size. Therefore, by complementing the amperometric recording of exocytosis with measurements using the intracellular electrochemical cytometry, *in situ* measurements of vesicle quantal size at live cells can be characterized and hence used to compare the fraction of vesicle content release from vesicles during exocytosis[26].

In this work, to investigate if the vesicle quantal size was affected by osmotic stress, we applied this technique for the quantification and evaluation of vesicle quantal size at cells exposed to isotonic and hypertonic conditions. As insertion of the nanotip electrode into the cytoplasm of a live cell is considered rather invasive, these experiments were only performed once per cell and were not repeated at the same cell. Therefore, these experiments are preferentially performed as individual measurements from groups of randomly selected cells exposed to an isotonic and a hypertonic extracellular environment. To compare changes in vesicle quantal size as measured by intracellular electrochemical cytometry to alterations in the amount neurotransmitter released at exocytosis, one should consider matching the experimental conditions of intracellular recording and also perform amperometry recording of exocytosis at separate random cells. If studies are performed on the same single cell, it is important in these experimental protocols to also consider the influence of consecutive $BaCl_2$ stimulations, and to ensure the experimental conditions match those used for intracellular cytometry measurements. It is also worth noting that it is difficult to control the exact placement and depth of the electrode when is inserted into a cell. Thus, each cell provides a random sample for vesicle quantal size analysis. In addition, probing vesicle quantal size using this method does not distinguish differences in, for instance, vesicle maturity and therefore also might add variation to measurements. Intracellular experiments showed that vesicle quantal size was reduced at cells exposed to extracellular osmotic pressure. The relative reduction in quantal size detected by this method was compared to the observations of relative changes in neurotransmitter release during exocytosis. This study found that the relative decline in quantal size was on the same order as the drop in neurotransmitter release at cells sensing osmotic stress.

Copyright © 2018 Journal of Visualized Experiments

To verify if osmotic stress was altering the vesicle neurotransmitter concentration, the vesicle volume was evaluated using TEM imaging analysis for chemically fixed chromaffin cells that had been exposed to isotonic and hypertonic conditions. TEM imaging analysis showed that vesicles shrink when cells are exposed to an osmotic shock and that the relative reduction in size is adjusted together with the vesicle quantal size to maintain a constant neurotransmitter concentration[24]. The TEM image analysis, which provides nanometer image resolution can distinguish the two phases, the dense core protein matrix and the surrounding halo solution inside LDCVs, and thus makes it possible to determine the volume of dense core protein matrix and calculate the volume of the halo solution. From this analysis, the decline in vesicle volume was determined to be mainly related to a diminishing volume from the halo solution surrounding the dense core protein matrix[24].

In summary, this study presents a protocol demonstrating how three analytical methods is combined, and allows characterization of secretory vesicles before neurotransmitter release and what are released from these vesicles when cells are triggered to exocytosis, to gain a better understanding of how secretory vesicles and cell functions like exocytosis is affected by extracellular stress. This protocol might also be used to help answer questions on how neurotransmitter release at exocytosis and vesicle quantal size is affected by other alterations in the physical environment or by potential drugs that may affect secretory cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank the Swedish Research Council (349-2007-8680) for funding and Dalsjöfors Kött AB (Dalsjöfors, Sweden) for donation of bovine adrenal glands.

References

1. Albillos, A. *et al.* The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature.* **, 389** (6650), 509-512 (1997).
2. Dunevall, J. *et al.* Characterizing the Catecholamine Content of Single Mammalian Vesicles by Collision-Adsorption Events at an Electrode. *J Am Chem Soc.* **137** (13), 4344-4346 (2015).
3. Crivellato, E.; Nico, B.; Ribatti, D., The chromaffin vesicle: advances in understanding the composition of a versatile, multifunctional secretory organelle. *Anat Rec.* **291** (12), 1587-1602 (2008).
4. Morris, S.; Schultens, H.; Schober, R., An osmometer model for changes in the buoyant density of chromaffin granules. *Biophys J.* **20** (1), 33 (1977).
5. Winkler, H.; Smith, A. D., The chromaffin granule and the storage of catecholamines. *Handbook of Physiology.* **6** (7), 321-399 (1975).
6. Phillips, J.; Allison, Y.; Morris, S., The distribution of calcium, magnesium, copper and iron in the bovine adrenal medulla. *Neurosci.* **2** (1), 147-152 (1977).
7. Estévez-Herrera, J. *et al.* ATP: The crucial component of secretory vesicles. *Proc Natl Acad Sci.* **2016, 113** (28), E4098-E4106. (2016).
8. Machado, J. D.; Camacho, M.; Alvarez, J.; Borges, R., On the role of intravesicular calcium in the motion and exocytosis of secretory organelles. *Commun integr biol.* **2** (2), 71-73 (2009).
9. Toll, L.; Howard, B. D., Role of Mg2+ ion-activated ATPase and a pH gradient in the storage of catecholamines in synaptic vesicles. *Biochemistry.* **17** (13), 2517-2523 (1978).
10. Terland, O.; Flatmark, T., Ascorbate as a natural constituent of chromaffin granules from the bovine adrenal medulla. *FEBS letters.* **59** (1), 52-56 (1975).
11. Camacho, M.; Machado, J. D.; Montesinos, M. S.; Criado, M.; Borges, R., Intragranular pH rapidly modulates exocytosis in adrenal chromaffin cells. *J Neurochem.* **96** (2), 324-334 (2006).
12. Jankowski, J. A.; Schroeder, T. J.; Ciolkowski, E. L.; Wightman, R. M., Temporal characteristics of quantal secretion of catecholamines from adrenal medullary cells. *J Biol Chem.* **268** (20), 14694-14700 (1993).
13. Daniels, A.; Williams, R.; Wright, P., The character of the stored molecules in chromaffin granules of the adrenal medulla: a nuclear magnetic resonance study. *Neurosci.* **3** (6), 573-585 (1978).
14. Borges, R.; Travis, E. R.; Hochstetler, S. E.; Wightman, R. M., Effects of external osmotic pressure on vesicular secretion from bovine adrenal medullary cells. *J Biol Chem.* **272** (13), 8325-8331 (1997).
15. Troyer, K. P.; Mundorf, M. L.; Fries, H. E.; Wightman, R., Separating vesicle fusion and exocytosis in hypertonic conditions. *Ann NY Acad Sci.* **971** (1), 251-253 (2002).
16. Brodwick, M. S.; Curran, M.; Edwards, C., Effects of osmotic stress on mast cell vesicles of the beige mouse. *J Membrane Biol.* **1992, 126** (2), 159-169 (1992).
17. Amatore, C.; Arbault, S.; Bonifas, I.; Lemaitre, F.; Verchier, Y., Vesicular exocytosis under hypotonic conditions shows two distinct populations of dense core vesicles in bovine chromaffin cells. *Chem Phys Chem.* **8** (4), 578-585 (2007).
18. Hampton, R. Y.; Holz, R. W., Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *J Cell Biol.* **96** (4), 1082-1088 (1983).
19. Troyer, K. P.; Wightman, R. M., Temporal separation of vesicle release from vesicle fusion during exocytosis. *J Biol Chem.* **2002, 277** (32), 29101-29107 (2002).
20. Pihel, K.; Travis, E. R.; Borges, R.; Wightman, R. M., Exocytotic release from individual granules exhibits similar properties at mast and chromaffin cells. *Biophys J.* **71** (3), 1633 (1996).
21. Jankowski, J. A.; Finnegan, J. M.; Wightman, R. M., Extracellular ionic composition alters kinetics of vesicular release of catecholamines and quantal size during exocytosis at adrenal medullary cells. *J Neurochem.* **63** (5), 1739-1747 (1994).
22. Leszczyszyn, D. J. *et al.* Nicotinic receptor-mediated catecholamine secretion from individual chromaffin cells. Chemical evidence for exocytosis. *J Biol Chem.* **265** (25), 14736-14737 (1990).

Copyright © 2018 Journal of Visualized Experiments

23. Wightman, R. *et al.* Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *P Natl Acad Sci.* **88** (23), 10754-10758 (1991).
24. Fathali, H.; Dunevall, J.; Majdi, S.; Cans, A.-S., Extracellular osmotic stress reduces the vesicle size while keeping a constant neurotransmitter concentration. *ACS Chemical Neuroscience.* (2017).
25. Bath, B. D. *et al.* Subsecond adsorption and desorption of dopamine at carbon-fiber microelectrodes. *Anal Chem.* **72** (24), 5994-6002 (2000).
26. Li, X.; Majdi, S.; Dunevall, J.; Fathali, H.; Ewing, A. G., Quantitative Measurement of Transmitters in Individual Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes. *Angew Chem Int Edit.* **54** (41), 11978-11982 (2015).
27. Zoski, C. G.; Mirkin, M. V., Steady-state limiting currents at finite conical microelectrodes. *Anal Chem.* **74** (9), 1986-1992 (2002).
28. Mosharov, E. V.; Sulzer, D., Analysis of exocytotic events recorded by amperometry. *Nat Methods.* **2** (9), 651-658 (2005).
29. Morris, J. K., A formaldehyde glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol.* **27**, 137-139 (1965).
30. Parsons, T. D.; Coorssen, J.; Horstmann, H.; Almers, W., Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. *Neuron.* **15** (5), 1085-1096 (1995).

Copyright © 2018 Journal of Visualized Experiments