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**Measurements of compensatory endocytosis by antibody internalization and quantification of endocytic vesicle distribution in adrenal chromaffin cells**

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**Running head :** Compensatory endocytosis assay in chromaffin cells

**Abstract**

Plasma membrane proteins are amenable to endocytosis assays since they are easily labeled by reagents applied in the extracellular medium. This has been widely exploited to study constitutive endocytosis or ligand-induced receptor endocytosis. Compensatory endocytosis is the mechanism by which components of secretory vesicles are retrieved after vesicle fusion with the plasma membrane in response to cell stimulation and a rise in intracellular calcium. Luminal membrane proteins from secretory vesicles are therefore transiently exposed at the plasma membrane. Here we described an antibody-based method to monitor compensatory endocytosis in chromaffin cells and present an image-based analysis to quantify endocytic vesicles distribution.

**Key words:** compensatory endocytosis, large dense core vesicle, neuroendocrine cells, chromaffin cells, internalization, dopamine- $\beta$ -hydroxylase

## 1. Introduction

A long standing question is how cell plasma membrane homeostasis is maintained especially in cells with high exchange rate between pools of intracellular vesicles and the plasma membrane. Neurons and neuroendocrine cells are heavily challenged to maintain cell to cell communication by releasing neurotransmitters and hormones through regulated exocytosis. To release their content upon cell stimulation, synaptic vesicles and secretory granules fuse with the plasma membrane resulting in a massive supply of vesicular membrane. Efficient compensatory endocytosis has to take place to maintain plasma membrane homeostasis and restore vesicular pools (1, 2). Several accurate and sensitive methods are widely used to study exocytosis including electrophysiological and electrochemical approaches that measure changes in electrical properties of the plasma membrane and detect the released molecules respectively (3–5). Live cell imaging with the use of pH-sensitive fluorescent proteins which shine when pH increases upon fusion pore opening represents an alternative approach (6, 7). Since plasma membrane recovers basal properties and fluorescent reporters are quenched upon acidification in endocytic compartments, such methods are however less suitable to follow the fate of proteins once the endocytic vesicle has formed. Therefore, the main challenge to study compensatory endocytosis is to specifically identify and track proteins that had been transiently exposed to the plasma membrane. Biochemical methods can give an overview of protein fate but often require large amounts of proteins that are not necessarily easily obtained, especially in model system with limited amount of cells such as mouse chromaffin cells for example. Adrenal medulla chromaffin cells have been an instrumental model to study exocytosis and compensatory endocytosis. In contrast to synaptic vesicles which size range is below the resolution limits of conventional light microscopy, secretory granules of chromaffin cells allows for single-vesicle exocytosis and endocytosis analysis

with conventional microscopy. By the use of specific antibodies that recognize luminal domain of secretory granule proteins, exocytic sites can be stained. Since vesicular trafficking and especially endocytosis requires energy to proceed, chilling cells at 4° after exocytic stimulation temporarily blocks endocytosis, providing time to specifically label proteins of interest with antibodies. By replacing cells at physiological temperature, compensatory endocytosis gets started and antibodies bound to vesicular proteins are uptaken. We are providing here protocols for time course experiments and immunofluorescence analysis of endocytic vesicle containing antibodies directed against the luminal domain of the dopamine- $\beta$ -hydroxylase (DBH), a secretory granule resident enzyme converting dopamine into noradrenaline. These protocols give access to spatio-temporal measurement of compensatory endocytosis in bovine chromaffin cells.

## **2. Materials**

### **2.1 Equipment**

1. 12 mm coverslips cleaned and sterilized (*see Note 1*).
2. Tissue culture well plates with a diameter able to accept coverlips (typically 24 well plates or 4 well plates with diameter of 15 mm)
3. Ice bucket to transfer plates or maintain coverslips in plates at 4°C to stop vesicular trafficking during antibodies incubation.
4. Hot plate is used to maintain cells at 37°C during cell stimulation and endocytosis time-course experiments.
5. Forceps to transfer coverlips between plates.
6. Confocal microscope
7. Open source Image analysis software (*see Note 2*).
8. Primary chromaffin cells cultured on coverslips (*see Note 3*).

## 2.2 Solution and reagents

1. Locke's solution: 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, 11 mM glucose, 0.57 mM ascorbic acid, and 15 mM HEPES, pH=7.5.
2. Nicotine solution: 1 M nicotine stock is prepared in Locke's solution and conserved on ice protected from light. 10 µM nicotine in Locke's solution is freshly prepared and warmed up to 37°C just before use.
3. High potassium Locke's solution: 85,7 mM NaCl, 59 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, 11 mM glucose, 0.57 mM ascorbic acid, and 15 mM HEPES, pH=7.5.
4. Phosphate buffer saline: 1 mM KH<sub>2</sub>PO<sub>4</sub>, 155 mM NaCl, 3 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4
5. Fibronectin from bovine plasma for coverslip coating: cleaned coverslips are incubated at least 2 hours at 37°C in 50 µg/ml fibronectin diluted in water.
6. Cell culture medium: bovine chromaffin cells are maintained in DMEM supplemented with 10% fetal bovine serum previously heat-inactivated for 30 min at 56°C, 1% glutamine, 1% cytosine arabinoside and 0.1% fluorodeoxyuridine solutions to prevent fibroblasts proliferation.
7. Bovine serum albumin (BSA): 0.2 % (w/v) BSA diluted in Locke's solution (*see Note 4*).
8. Anti-dopamine β-hydroxylase (DBH) antibodies (*see Note 5*).
9. Paraformaldehyde (PFA) solution: 16 % PFA stock solution is diluted at 4% in PBS to fix cells.
10. Permeabilization solution: 0.1% (v/v) Triton X-100 diluted in PBS.
11. Fluorophore-conjugated secondary antibodies (*see Note 6*).

12. Coverslips mounting medium: dissolve 1 g of Mowiol 4-88 in 4 ml PBS by gentle steering for 16 h at room temperature. Add 2 ml anhydrous glycerol and steer for additional 16 h. Centrifuge for 15 min at 13000 g, aliquot and store at 4°C for several months.

### **3. Methods**

#### **3.1 Anti-DBH antibodies internalization assay**

1. Plate freshly isolated chromaffin cells on fibronectin coated coverslips at 1250 cells/mm<sup>2</sup>. Assay can be performed from 48 hours to 10 days after plating (*see Note 7*).
2. Place cells on heated plate at 37°C. Replace cell culture medium by pre-warmed Locke's solution. Incubate for 10 min at 37°C (*see Note 8*).
3. Stimulate cells for 2 min at 37°C by adding 10 µM nicotine solution in Locke's solution. Alternatively, cells may be stimulated by a depolarizing solution of 59 mM potassium (see solution and reagent n°3).
4. Transfer cells on ice and wash once with ice-cold Locke's solution.
5. Remove Locke's solution and add ice-cold anti-DBH antibodies diluted in Locke's containing 0.2% BSA. Incubate for 30 min on ice.
6. Wash cells twice with ice cold Locke's solution and transfer coverslips in 37°C pre-warmed Locke's solution to initiate endocytosis.
7. Incubate for the required time (*see Note 9*) before transferring back cells on ice to stop all vesicular trafficking. Maximum endocytosis is considered to be achieved after 10 min at 37°C.
8. Remove Locke's solution and add 4% ice-cold PFA to fix cells for 10 min at room temperature.
9. Wash 3 times with PBS at room temperature and permeabilize cells for 12 min in 0.1% Triton X-100 solution.

10. Wash 3 times with PBS and proceed with anti-DBH antibodies detection by adding corresponding fluorescent secondary antibodies (*see Note 6*) in PBS containing 3% BSA for 30 min at room temperature.

11. Wash 3 times with PBS and mount coverslips in Mowiol 4-88. Store at 4°C until observation.

### **3.2 Image acquisition and analysis**

1. Cells are observed under a confocal microscope. Acquisition is performed according to Nyquist parameters with high numerical aperture objectives (x63, NA1.4). Pixel resolution should be around 90 nm for 488 nm excitation wavelengths and intensity has to be carefully checked to avoid saturation. Optical section must be done at the equatorial plane of round cells to get accurate measurement of endocytosis.

2. Image files can be processed with open source softwares like ImageJ or Icy. We will describe here the steps using Icy software which has the advantages of keeping region of interest (ROI) information in xml files associated to tiff format. In addition, Icy allows for automated image analysis without any coding knowledge, thanks to graphical programming (Fig.1). Some features of ImageJ are embedded in Icy such as basic watershed segmentation and Euclidean distance map that are used for image analysis. Icy protocol is available on Icy website (<http://icy.bioimageanalysis.org/>) or upon request.

3. The first step is to delineate cells edge. Phase contrast image or if available, cell membrane (*see Note 10*) or actin staining can be used. Cell delineation can be done manually (*Area tool*) or if staining is well contrasted, specific plugins can be used to find cell edges (*HK-Means*, *Active contours*). ROI are then filled in with *Fill holes in ROI* plugin.

4. DBH staining is then segmented using wavelet transform decomposition of original image to efficiently detect DBH spots (**8**). This is integrated in *Spot detector* plugin of Icy (Fig.1).

Parameters have to be empirically defined and binary image compared to original image (*see Note 11*).

5. Since detections are exported as ROI on the original pictures, basic measurements such as mean spot intensity, spot number and size using *ROI measurements* plugin can be easily obtained. Such parameters are, for example, useful to compare exocytosis efficiency between two conditions (*9, 10*)

6. To measure the efficiency of endocytosis, the position of endocytosed DBH antibodies at different incubation time at 37°C has to be defined. To do so, ROI corresponding to cell area is converted into Euclidean distance map (EDM) which gives a gray intensity to each pixel as the function of their distance to the edge of the ROI (Fig.2A). Consequently, pixel intensity increases as it separates from the ROI edge. Then, ROI corresponding to DBH staining are transferred onto EDM picture (Fig.2A). Mean intensity of each ROI are measured and exported into Excel files. By compiling all files, distribution of spots intensity can be obtained. An example of DBH-positive spot distribution for a single cell is shown (Fig.2B and C). A distance threshold can be applied to estimate the percentage of stained vesicles remaining at a specific distance from the cell edge (*9–11*).

#### **4. Notes**

1. Coverslips are vigorously washed with detergent solution and extensively rinsed with distilled H<sub>2</sub>O. Coverslips are transferred into pure ethanol and dried out by wiping them with clean soft tissues.

2. Image analysis are performed with open source Icy software which integrates ImageJ (<http://icy.bioimageanalysis.org/>).

3. Procedures to culture primary chromaffin cells from different organisms including cow, rat, mouse have been carefully described previously (*12*) and in this issue (*13*)

4. 0.2 % (w/v) BSA diluted in Locke's solution is used on live cells during antibody incubation to minimize non-specific binding of antibodies.
5. Rabbit anti-dopamine  $\beta$ -hydroxylase (DBH) antibodies we used was as described previously (*14*). A monoclonal anti-DBH antibody (Millipore, clone 4F10.2) is suitable when rabbit polyclonal antibodies are used for co-staining experiments. Each antibody lot has to be checked to adjust effective dilution. Other sources of antibody have not been tested.
6. All secondary antibodies coupled to fluorescent molecule directed against the primary antibody can be used. We routinely use Alexa-conjugated goat anti-rabbit antibodies at 1  $\mu$ g/ml diluted in PBS containing 3% BSA.
7. Since cells tend to spread out and to adopt an elongated and flattened morphology with time in culture, we recommend performing experiments within the first 5 days of cell culture to get accurate and reproducible measurements of compensatory endocytosis.
8. Chromaffin cells in culture are highly sensitive to mechanical stress. Manipulation and changes in medium must be gentle to avoid mechanical cell stimulation and limit DBH staining in resting state.
9. After 2 to 3 min incubation at 37°C, most DBH staining remains close to the plasma membrane. It is therefore difficult to assess whether DBH is internalized or still at the cell surface. Acidic buffer might be used to strip off cell surface antibodies. Conversely, at 5 min, most DBH staining is seen inside the cell at distance from the plasma membrane.
10. For accurate cell delineation, plasma membrane proteins can be biotinylated by amine reactive biotin (Sulfo NHS-SS-Biotin) and then revealed using fluorescent streptavidin. Plasma membrane protein biotinylation can be done before or after cell fixation but cell contour are better define when cells are biotinylated before fixation. Following anti-DBH endocytosis assay, cells are incubated an additional 30 min on ice in PBS containing

120 µg/ml Sulfo NHS-SS-Biotin. Cells are rinsed twice in ice cold PBS and fixed. Biotinylated proteins are revealed by incubating cells with fluorescently-labelled streptavidin.

11. Typically, at 90 nm pixel resolution, sensitivity can be set to scale 2 and threshold between 30 and 70 to obtain good results. To get the best accuracy between number of spots before and after segmentation, ImageJ watershed is applied to separate spots that are too close to be resolved by spot detector.

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