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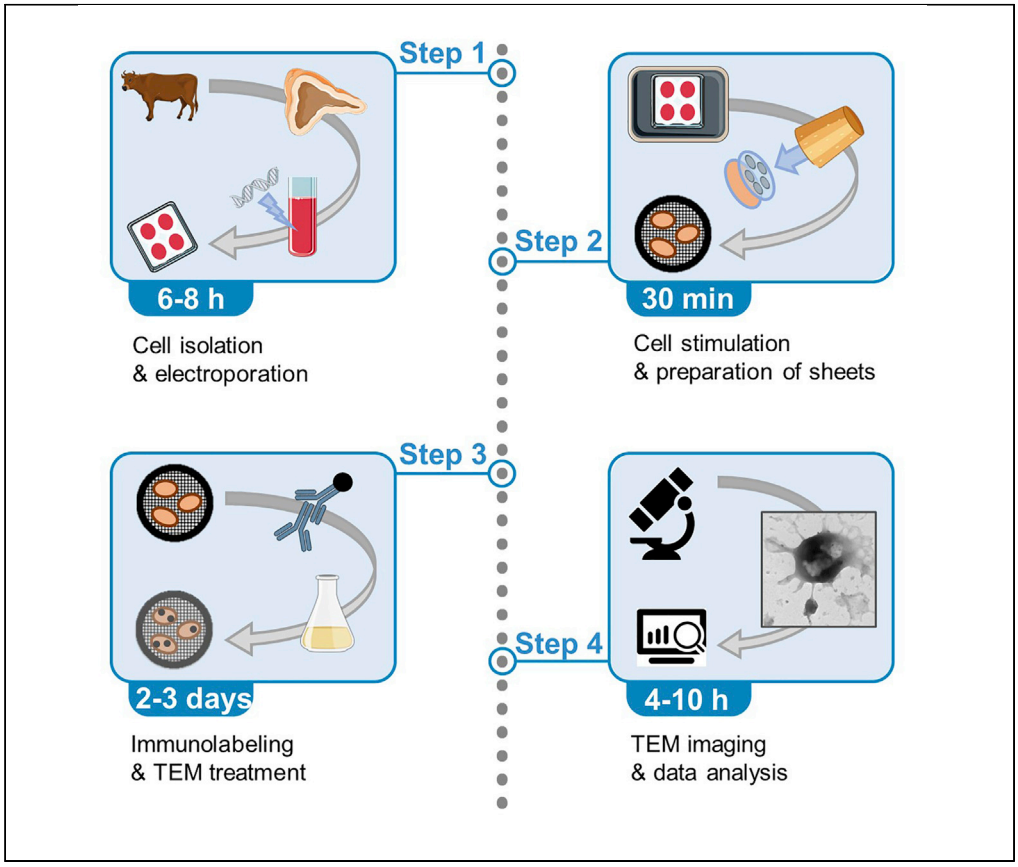
Protocol for electron microscopy ultrastructural localization of the fusogenic lipid phosphatidic acid on plasma membrane sheets from chromaffin cells



The glycerophospholipid phosphatidic acid (PA) is a key player in regulated exocytosis, but little is known about its localization at the plasma membrane. Here, we provide a protocol for precisely determining the spatial distribution of PA at exocytotic sites by electron microscopy. Using primary bovine chromaffin cells expressing a PA sensor (Spo20p-GFP), we describe the process for cell stimulation and detergent-free preparation of plasma membrane sheets. The protocol can be applied to other cell models and to distinct membrane lipids.

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Highlights

Expression of a genetically encoded sensor for phosphatidic acid in chromaffin cells

Preparation of membrane sheets from secretory cells

Detection of phosphatidic acid sensors on plasma membrane sheets near exocytosis sites

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Protocol for electron microscopy ultrastructural localization of the fusogenic lipid phosphatidic acid on plasma membrane sheets from chromaffin cells

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SUMMARY

The glycerophospholipid phosphatidic acid (PA) is a key player in regulated exocytosis, but little is known about its localization at the plasma membrane. Here, we provide a protocol for precisely determining the spatial distribution of PA at exocytotic sites by electron microscopy. Using primary bovine chromaffin cells expressing a PA sensor (Spo20p-GFP), we describe the process for cell stimulation and detergent-free preparation of plasma membrane sheets. The protocol can be applied to other cell models and to distinct membrane lipids. For complete details on the use and execution of this protocol, please refer to Tanguy et al. (2020).

BEFORE YOU BEGIN

The protocol below describes the steps to precisely localize phosphatidic acid (PA) on plasma membrane sheets using a genetically encoded specific probe. However, we have also used variations of this protocol either with transfected or non-transfected cells to study the spatial distribution of other membrane lipids involved in exo-endocytosis processes (Gabel et al., 2015; Ory et al., 2013; Umbrecht-Jenck et al., 2010).

Prepare cleaned and sterilized coverslips

Timing: at least 7 h

1. Clean 12-mm circle glass coverslips.
   a. Place coverslips in a 50 mL Falcon® tube and add 2 mL of any commercial multi-usage detergent solution (e.g., Mr. Clean) diluted in 40 mL of ultrapure water.
   b. To wash coverslips, fix the tube horizontally on an orbital shaker and agitate for 4 h at room temperature.
   c. Vigorously rinse coverslips several times with ultrapure water.
   d. Discard rinsing solution and cover coverslips with 40 mL of pure ethanol.
   e. Seal the tube with parafilm.

Note: Coverslips can be stored in ethanol indefinitely at room temperature.

2. Take coverslips one by one with a forceps and wipe with soft tissues (e.g., Kimtech® wipes).
3. Place coverslips on a clean tissue to dry out.
4. Transfer dry coverslips to a clean glass dish and cover the dish with aluminum foil.
5. Sterilize coverslips in an oven at 200°C for 2 h and store them at room temperature until use.

Prepare Formvar-coated TEM grids

Timing: 2–3 h (for 100 grids)

6. Clean glass slides using ultrapure water and wipe with soft tissues (e.g., Kimtech® wipes).

Note: One simple 5–10 s water rinse is enough to remove potential dust or dirt. Do not over-clean nor use ethanol, otherwise Formvar films will not release from the slide.

7. Prepare 100 mL of 0.5% Formvar solution in chloroform.

Note: Due to potential permeation of chloroform solvent through the plastic, this step needs to be done in a glass bottle.

8. Fill a container with 5 cm deep 0.5% Formvar using a thistle tube.

Note: Keep the container covered with a glass cover as much as possible to prevent evaporation of chloroform and work under a chemical fume hood to prevent inhalation.

9. Place a slide vertically in Formvar and let sit for around 15 s.
10. Slowly remove the slide from Formvar in 3–5 s and let it drain above the solution until the Formvar film on the slide tends to be gold-colored.

⚠ CRITICAL: Retraction speed and draining duration will determine the thickness of the Formvar film, noticeable by its color (from light gray to golden). A gold-colored film is required for coating grids, as gray films are usually too thin to undertake the pressure applied to prepare membrane sheets.

11. Remove the slide from container and complete drying by placing it vertically on filter paper.
12. Use a razor blade to scrape edges of the slide and around 0.5 cm from the bottom of the slide.
13. Fully remove the Formvar film from the slide.
    a. Fill a container with sterile ultrapure water.
    b. Breath moist air onto the slide.
    c. Slowly immerse slide into the water at around 45° angle and let float off film.

Note: Gently pull the Formvar film from the slide with forceps if needed.

14. Coat 3.05 mm diameter 200 mesh nickel grids (Electron Microscopy Sciences, Cat#M200-Ni) with Formvar film (5 grids/coverslip).
    a. Place 5 grids with mat side down onto the film.
    b. Lower a clean coverslip over the grids at around 45° angle with a forceps.
    c. Rapidly push down and turn the coverslip to hold Formvar-coated grids against the coverslip.

⚠ CRITICAL: Use of copper grids is not recommended, as copper could prevent immunolabeling and may damage live cells during preparation of membrane sheets.

15. Remove excess water from the coverslip with filter paper and place in a Petri dish lined with filter paper to dry.

16. Coat the grids with a carbon thread evaporation device (Leica microsystems, Cat#CED030).
    a. Fix a carbon fiber (double-braided carbon wires) on the mounting holder on the top of the vacuum chamber.
    b. Put the dish under the chamber and adjust height to a 50 mm working distance, to obtain a 10-nm thick carbon film.
    c. Degas the chamber and apply high voltage to disperse a double carbon thread on the Formvar-coated grids.
17. Seal the dish and store at room temperature until use.

Prepare solutions

Timing: 1–2 h

18. Prepare the necessary solutions according to the "materials and equipment" section.

⚠ CRITICAL: Use sterile ultrapure water (e.g., purified deionized water, 18 MΩ-cm at 25°C).

Note: We recommend using freshly prepared solutions (≤24 h before experiments) to prevent sample contamination by microorganisms.

19. If needed, prepare the transfection buffer with the reagents available in a box of "Basic Nucleofector Kit for Primary Mammalian Neurons".
    a. Add all the "Supplement 1" (0.5 mL) to the vial of "Nucleofector Solution for Primary Mammalian Neurons" (2.25 mL).
    b. Gently mix the vial.
    c. Store the vial sealed at 2°C–8°C until used, for up to 3 months.

Note: The transfection buffer must be prepared in sterile conditions under a laminar flow cabin in a cell culture room.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP	Abcam	Cat#ab290; RRID: AB_303395
15-nm Gold particle-conjugated goat anti-rabbit IgG	Aurion	Cat#815.011
Biological samples		
Bovine adrenal glands	Municipal slaughterhouse of Haguenau	N/A
Chemicals, peptides, and recombinant proteins		
Fibronectin from bovine plasma	Sigma-Aldrich	Cat#F1141
Dulbecco's modified Eagle's medium (DMEM) - low glucose	Sigma-Aldrich	Cat#D5546
Fetal bovine serum (FBS)	Gibco	Cat#10270-106
L-Glutamine (200 mM)	Gibco	Cat#25030-123
Cytosine arabinoside	Sigma-Aldrich	Cat#C6645
5-Fluorodeoxyuridine	Sigma-Aldrich	Cat#F0503
Primocin (50 mg/mL)	InvivoGen	Cat#ant-pm-1
D-Glucose	Sigma-Aldrich	Cat#G7021
L-Ascorbic acid	Sigma-Aldrich	Cat#A4544
NaOH	Sigma-Aldrich	Cat#S8045
KOH	Sigma-Aldrich	Cat#P5958

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NaCl	Sigma-Aldrich	Cat#S5886
KCl	Sigma-Aldrich	Cat#P5405
$CaCl_2$	Sigma-Aldrich	Cat#C7902
$MgSO_4$	Sigma-Aldrich	Cat#M2643
KH_2PO_4	Sigma-Aldrich	Cat#P5655
EDTA	Sigma-Aldrich	Cat#E6758
HEPES	Sigma-Aldrich	Cat#H4034
Formvar	Agar Scientific	Cat#AGR1201
Chloroform	Sigma-Aldrich	Cat#319988
Paraformaldehyde (PFA) (16%)	Electron Microscopy Sciences	Cat#50-980-487
PBS (10×)	Euromedex	Cat#ET330
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A7030
Acetylated BSA (BSA-c) (10%)	Aurion	Cat#900.099
Glutaraldehyde (50%)	Sigma-Aldrich	Cat#G7651
Osmium tetroxide (OsO_4) (4%)	Electron Microscopy Sciences	Cat#RT19150
Sodium cacodylate trihydrate	Sigma-Aldrich	Cat#20840
HCl	Sigma-Aldrich	Cat#320331
Ethanol	Sigma-Aldrich	Cat#32221
Hexamethyldisilazane (HMDS) reagent grade ≥ 99%	Sigma-Aldrich	Cat#440191
Critical commercial assays		
Basic Nucleofector™ Kit for Primary Mammalian Neurons	Lonza	Cat#VVPI-1003
Recombinant DNA		
Plasmid: Spo20p-GFP	Nicolas Vitale (Kassas et al., 2017)	N/A
Software and algorithms		
Adobe Photoshop	Adobe	https://www.adobe.com
Microsoft Excel	Microsoft Office	https://www.microsoft.com
SigmaPlot 13	Systat Software	https://systatsoftware.com

MATERIALS AND EQUIPMENT

Transfection

Freshly isolated chromaffin cells and DMEM media are maintained at 37°C in a dry bath (Eppendorf® ThermoStat Plus). Cells are electroporated using the Nucleofector II Device (Lonza, Cat#AAB-1001) and plated on fibronectin-coated (see next section for details) 12-mm circle coverslips (Marienfeld, Cat#0111520) disposed in 4-well tissue culture plates (Nunc 4-well dishes, ThermoScientific, Cat#179830).

Alternatives: Coverslips can also be placed in 24-well plates.

Alternatives: We have also used this protocol to obtain plasma membrane sheets from PC12 cell line (rat adrenal pheochromocytoma cells) expressing Spo20p-GFP, with similar outcome. To transfect PC12 cells, we use Lipofectamine 2000 transfection reagent (Invitrogen, Cat#11668019) according to manufacturer's recommendations.

Cell stimulation and plasma membrane sheet preparation

The following materials and equipment are needed for these steps:

- An epifluorescence microscope with a dichroic filter (505–530 nm).
- A hot plate to maintain cells at 37°C during cell stimulation.
- Plastic Pasteur pipettes for washing steps.
- A pair of fine-tip forceps for plasma membrane sheets preparation.

- Homemade Formvar-coated TEM grids carried on coverslips.
- A cork of 10 mm of diameter.
- 4-well dishes.

DMEM-D medium		
Reagent	Final concentration	Amount
DMEM	n/a	440 mL
FBS	10%	50 mL
L-glutamine (200 mM)	4 mM	10 mL
Total	n/a	500 mL

DMEM-C2 medium		
Reagent	Final concentration	Amount
DMEM	n/a	427 mL
FBS	10%	50 mL
L-glutamine (200 mM)	4 mM	10 mL
Primocin (50 mg/mL)	200 µg/mL (2×)	2 mL
Cytosine arabinoside	20 µM (2×)	10 mL
5-fluorodeoxyuridine	20 µM (2×)	1 mL
Total	n/a	500 mL

Note: FBS must be previously decomplemented for 30 min at 56°C and stored at −20°C.

Note: DMEM media are freshly prepared during cell isolation according to amounts needed and kept at room temperature before use.

Preparation of stock solutions (stored at −20°C)

- Cytosine arabinoside 1 mM: Dilute 30 mg in 100 mL of DMEM, filter-sterilized and aliquot.
- 5-fluorodeoxyuridine 10 mM: Dilute 24.6 mg in 10 mL of Locke's solution, filter-sterilized and aliquot.

Locke's solution		
Reagent	Final concentration (mM)	Amount
D-Glucose	11	99 mg
Ascorbic acid	0.57	5 mg
NaCl (1 M)	140	7 mL
KCl (1 M)	4.7	235 µL
$CaCl_2$ (1 M)	2.5	125 µL
$MgSO_4$ (1 M)	1.2	60 µL
KH_2PO_4 (1 M)	1.2	60 µL
EDTA (10 mM)	0.01	50 µL
HEPES (1.5 M)	15	500 µL
Ultrapure H_2O	n/a	For 50 mL final
Total	n/a	50 mL

Note: Adjust the pH to 7.5 with NaOH 5 M and store at 2°C–8°C.

High K^+ depolarizing solution		
Reagent	Final concentration (mM)	Amount
D-Glucose	11	99 mg
Ascorbic acid	0.57	5 mg
NaCl (1 M)	85.7	4.35 mL
KCl (1 M)	59	2.89 mL
$CaCl_2$ (1 M)	2.5	125 µL
$MgSO_4$ (1 M)	1.2	60 µL
KH_2PO_4 (1 M)	1.2	60 µL
EDTA (10 mM)	0.01	50 µL
HEPES (1.5 M)	15	500 µL
Ultrapure H_2O	n/a	For 50 mL final
Total	n/a	50 mL

Note: Adjust the pH to 7.2 with KOH 5 M and store at 2°C–8°C.

⚠ CRITICAL: Locke's and high K^+ depolarizing solutions can be used for up to 2 weeks after preparation but should be replaced in case of signs of microorganism growth and detectable precipitates.

Preparation of stock solutions (stored at 2°C–8°C)

- NaCl 1 M: Dilute 58.44 g in 1 L of water.
- KCl 1 M: Dilute 3.73 g in 50 mL of water.
- $CaCl_2$ 1 M: Dilute 1.5 g in 10 mL of water.
- $MgSO_4$ 1 M: Dilute 2.46 g in 10 mL of water.
- KH_2PO_4 1 M: Dilute 1.36 g in 10 mL of water.
- EDTA 10 mM: Dilute 37 mg in 10 mL of water.
- HEPES 1.5 M: Dilute 35.7 g in 100 mL of water.

Blocking solution		
Reagent	Final concentration	Amount
BSA	1% (m/v)	0.5 g
BSA-c (10%)	0.1%	0.5 mL
PBS (10×)	1×	5 mL
Ultrapure H_2O	n/a	For 50 mL final
Total	n/a	50 mL

⚠ CRITICAL: Blocking solution should be stored at −20°C to prevent growth of microorganisms.

Paraformaldehyde fixative solution		
Reagent	Final concentration	Amount
PFA (16%)	2%	1.25 mL
PBS (10×)	1×	1 mL
Ultrapure H_2O	n/a	7.75 mL
Total	n/a	10 mL

Glutaraldehyde fixative solution		
Reagent	Final concentration	Amount
Glutaraldehyde (50%)	2.5%	0.5 mL
Sodium cacodylate buffer (0.1 M)	n/a	9.5 mL
Total	n/a	10 mL

Note: Fixative solutions must be prepared freshly before use and stored at 2°C–8°C until use.

Preparation of EM treatment solutions

- Sodium cacodylate buffer 0.1 M: Dilute 21.40 g of sodium cacodylate in 1 L of water, adjust pH to 7.4 with HCl 1N and store at 2°C–8°C.
- Osmium tetroxide (OsO_4) 0.5%: Dilute OsO_4 4% in the required volume of water (1:8) and store at 2°C–8°C.
- Ethanol solutions (25%, 50%, 70%, and 95%): Dilute ethanol 100% in water at the required concentration.
- HMDS - ethanol (50%/50%) solution: Dilute HMDS in the required volume of pure ethanol (1:1) just before use.

⚠ CRITICAL: Concentrated PFA, glutaraldehyde, and osmium tetroxide are irritant, very toxic, and possible carcinogenic reagents. Preparation of solutions should be done under a chemical fume hood to prevent inhalation.

Transmission electron microscopy (TEM) imaging

We use a Hitachi 7500 transmission electron microscope operated at 80 kV and equipped with an AMT Hamamatsu digital camera.

STEP-BY-STEP METHOD DETAILS

Coating of coverslips

Timing: 3–4 h (usually done simultaneously with cell isolation)

This step will enhance chromaffin cells adhesion to coverslips.

1. Dilute stock fibronectin solution (1 mg/mL) in water to obtain a fibronectin solution at 10 µg/mL.
2. Place cleaned and sterilized 12-mm coverslips in 4-well plates using sterile forceps.
3. Add 500 µL of 10 µg/mL fibronectin to each well containing a coverslip.

Note: Carefully spread the fibronectin solution to evenly cover the surface of the coverslips.

4. Incubate the plates at 37°C for at least 3 h.

Note: Coverslips incubation in coating solution should be completed by the end of chromaffin cell isolation.

5. Remove fibronectin from the wells just before adding medium.

Preparation of cells

Timing: 4–6 h (depending on the number of adrenal glands)

This step will provide primary chromaffin cells after isolation from bovine adrenal glands.

6. Isolate chromaffin cells from fresh bovine adrenal glands according to the protocol previously described (Thahouly et al., 2021).
7. Suspend the needed amount of freshly isolated chromaffin cells into 10 mL of DMEM-D medium in a 50 mL Falcon® tube.

   Note: We use 7.5 × 10^6 cells for one electroporation condition, but it is possible to reduce this amount to 5 × 10^6 cells if needed.

8. Maintain cells at 37°C until use (typically within 1 h).

   ⚠ CRITICAL: Electroporation should be performed as soon as possible after cell isolation to optimize cell viability and transfection efficiency.

Transfection of bovine chromaffin cells

Timing: 20–30 min

This step will allow transient cell expression of the PA probe.

9. Add 400 μL of DMEM-D medium to each well of 4-well plates containing a fibronectin-coated coverslip and place the plates at 37°C, 5% CO_2.
10. Equilibrate the transfection buffer (Nucleofector Solution for Primary Mammalian Neurons containing Supplement 1) at room temperature.

    ⚠ CRITICAL: Supplemented Nucleofector Solution can be used within 3 months and stored at 2°C–8°C to optimize cell viability and transfection efficiency.

11. Thaw an aliquot of Spo20p-GFP plasmid at room temperature and prepare 2 μg of plasmid into a 1.5 mL Eppendorf tube.

    ⚠ CRITICAL: To optimize transfection efficiency, plasmids must be prepared with an endotoxin-free plasmid purification kit. The concentration of plasmid preparation can be determined by optical density measurement using a Nanodrop spectrometer (Thermo Fischer Scientific). Aliquot before storage at −20°C to avoid repeated freezing and thawing.

    Note: As a control to further estimate potential background labeling induced by GFP binding on plasma membrane, another sample of 7.5 × 10^6 cells should be electroporated with a GFP expression plasmid.

12. Centrifuge the previously prepared cell aliquots at 100 *g*, 5 min at room temperature.
13. Entirely discard the supernatant.
14. Gently resuspend the pellet with 100 μL of transfection buffer for 7.5 × 10^6 cells (troubleshooting 1).

    ⚠ CRITICAL: Resuspension must be done as gently as possible with a 1000 μL micropipette to preserve cell viability. Otherwise, the suspension may turn viscous, indicating DNA release.

    ⚠ CRITICAL: From this step, electroporation and plating should be done in less than 15 min to preserve cell viability.

15. Add 100 μL of suspended cells to each 1.5 mL tube containing plasmid and gently mix.

Note: One electroporation sample contains 7.5 × 10^6 cells, 1–3 μg plasmid DNA (2 μg for Spo20p-GFP) and 100 μL transfection buffer.

16. Transfer the mixture into an electroporation cuvette with a single-use pipette.

⚠ CRITICAL: Mixing and transfer must be done as gently as possible to prevent formation of air bubbles that can reduce electroporation efficiency. If needed, try to get rid of air bubbles by gently tapping the cuvette on a hard surface (troubleshooting 1).

17. Insert the cuvette into the Nucleofector device and electroporate cells using the Amaxa nucleofector program X-001.
18. Immediately after electroporation add 500 μL of DMEM-D medium to each cuvette.
19. Gently resuspend cells and collect suspension with a single-use pipette.

Note: Pre-warm the DMEM-D medium to 37°C before use.

20. Plate cells by adding 4–6 drops (each drop corresponding to ≈25 μL) to each well already containing 400 μL of warmed DMEM-D medium and mix gently.

Note: With a 600 μL cell suspension after one electroporation, we typically plate 4 wells.

21. Incubate cells at 37°C, 5% CO_2.
22. Between 4–6 h after plating, add 1:1 (vol to vol) of pre-warmed DMEM-C2 medium to each well (500–550 μL) and put cells back at 37°C, 5% CO_2.

⚠ CRITICAL: This step is critical for cell viability and helps to prevent contamination.

Note: DMEM-C2 medium contains the same components than DMEM-D medium plus 2× concentrated antibiotics (Primocin) to prevent growth of microorganisms and antimitotics (cytosine arabinoside and 5-fluorodeoxyuridine) to prevent growth of few fibroblasts remaining in the chromaffin cell culture. Adding DMEM-C2 instead of refreshing medium preserves cell adhesion and prevents detaching loosely attached cells.

Note: Pre-warm the DMEM-C2 medium to 37°C before use.

Stimulation of exocytosis and preparation of plasma membrane sheets

Timing: 30 min

The purpose of this step is to apply mechanical forces to break open cells after they were stimulated to trigger exocytosis or placed in resting condition.

23. Pre-warm the Locke's and high K^+ depolarizing solutions in a water bath set to 37°C.
24. Around 16–18 h after transfection, place cells onto a hot plate set at 37°C.

⚠ CRITICAL: Long-term expression of the probe Spo20p-GFP may induce cytotoxicity. In our hands, expression of Spo20p-GFP can be carried for up to 18 h after electroporation without significant cell death, but longer expression times are not recommended.

Note: We recommend to simultaneously perform the next steps with untransfected bovine chromaffin cells cultured on coverslips to assess unspecific binding of antibodies (background).

25. Aspirate medium from wells and wash cells 3 times with Locke's solution (1 mL per well) (troubleshooting 2).

Note: Chromaffin cells in culture are highly sensitive to mechanical stress. Manipulation and medium changes must be gentle to avoid mechanical cell stimulation.

Optional: Before stimulation, observe cells under an inverted epifluorescence microscope to ensure cell viability and evaluate transfection efficiency.

26. Stimulate exocytosis.
    a. Add 500 µL per well of high K^+ depolarizing solution (stimulated condition) or Locke's solution (resting condition).
    b. Incubate cells for 10 min at 37°C.

Note: Pre-warm the Locke's and high K^+ depolarizing solutions in a water bath set to 37°C.

Note: Treating each well every 60 s appears well-suited to accommodate further preparation of membrane sheets with several 4-well plates.

27. During incubation, prepare the material required for preparation of plasma membrane sheets (Figure 1A) as well as other 4-well plates for fixation of sheets by adding 400 µL of 2% ice-cold PFA per well.
28. Immediately after the end of stimulation, prepare plasma membrane sheets for each well of resting or stimulated chromaffin cells, according to the protocol previously described (Delavoie et al., 2021).
    a. Flip a coverslip carrying Formvar-coated EM grids onto cells (Figures 1B and 1C).
    b. Place the cork above the coverslips and slightly press for 20–25 s (Figure 1D).
    c. Rapidly dissociate the coverslips with 2 forceps and lift the one carrying grids (Figures 1E and 1F).

Note: This procedure promotes cell adhesion to the Formvar film to induce cell fracture by dissociation of the two coverslips, exposing cytoplasmic face of the upper cell membrane.

⚠ CRITICAL: Plasma membrane sheets must be prepared as quickly as possible (typically in less than 30 s) to preserve membrane organization.

29. Immediately transfer the coverslip carrying grids to a well containing 2% ice-cold PFA (Figure 1G) and fix sheets for 10 min at room temperature.
30. Remove fixative from wells and wash 6 times with 500 µL of PBS per well at room temperature.

⚠ CRITICAL: During washing steps, PBS changes must be very gentle to prevent detachment of Formvar film from the grids or detachment of the grids from the coverslip. If one grid completely pulls away from the coverslip, remove it and put it in another well to apply the following steps. These grids can be imaged if needed, but we preferentially use the grids still attached to the coverslip.

Pause point: Sheets can be stored in PBS at 4°C for few days until performing immunocytochemistry.

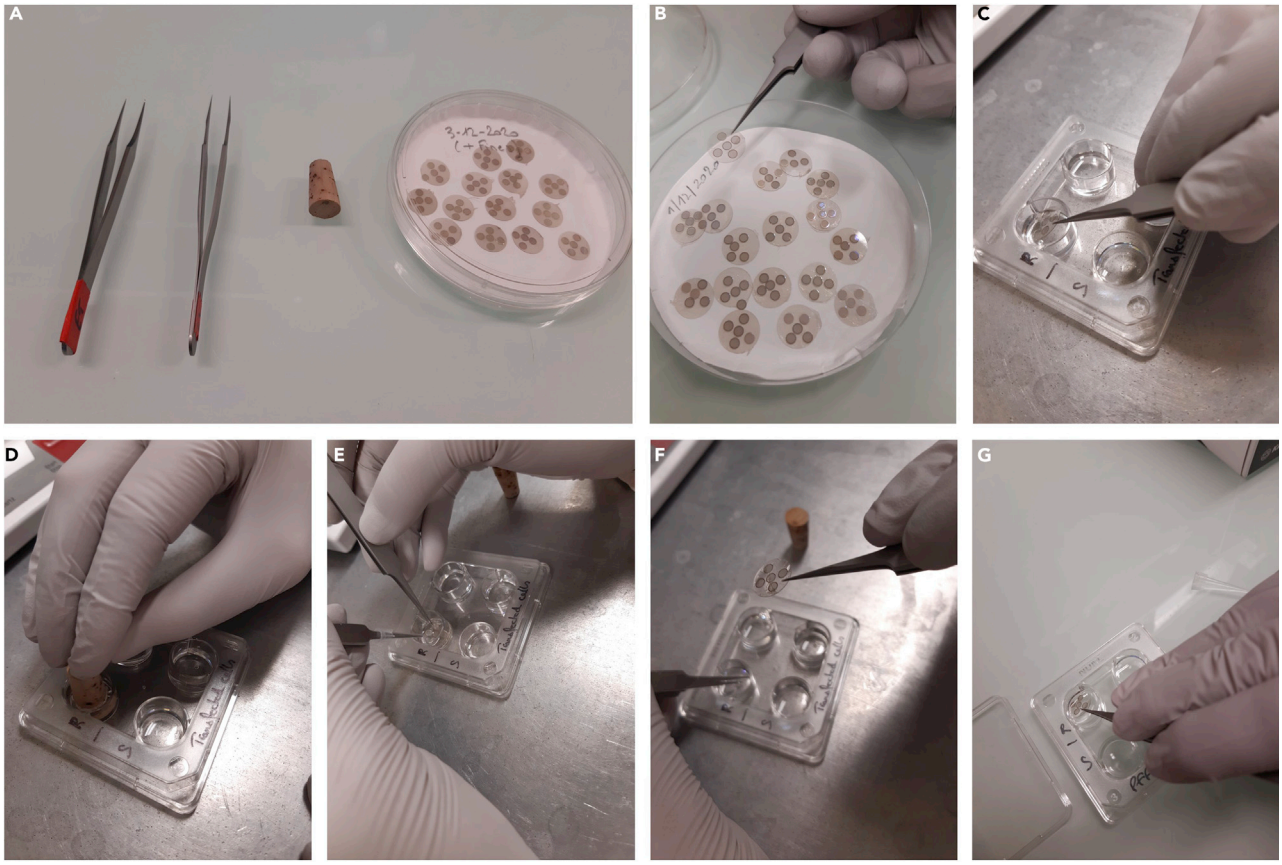

Figure 1. Preparation of plasma membrane sheets

(A) Prepare material needed during the 10 min stimulation step.

(B) Take a coverslip exposing Formvar-coated EM grids.

(C) Flip the coverslip onto cells.

(D) Place the cork in the center of the well and press during 25 s to allow cell adhesion to Formvar-coated grids.

(E) Rapidly recover the upper coverslips with forceps to break off cells.

(F) Lift the coverslip to expose membrane sheets on the upper side.

(G) Transfer the coverslip carrying grids in fixative.

Immunolabeling and sample treatment for electron microscopy

Timing: 2–3 days

The purpose of this step is to label GFP on plasma membrane sheets with specific antibodies and prepare the samples for TEM imaging.

31. Remove PBS from wells and incubate sheets with 400 µL of blocking solution per well overnight at 4°C.
32. Incubate sheets with primary antibodies (rabbit anti-GFP) (troubleshooting 3).
    a. Prepare a 1:100 dilution of the primary antibody in the blocking solution.
    b. Retrieve coverslips from wells one by one using a forceps and dispose them on raised supports in a humidity chamber (we use caps of Eppendorf® tubes glued to a Petri dish).
    c. Immediately add 50 µL of primary antibody dilution onto each coverslip.
    d. Add 2 mL of water in the dish to create a humidity chamber, seal, and incubate for 4 h at room temperature.

⚠ CRITICAL: Rapidly apply the antibody solution onto the grids to avoid sample drying and be careful to entirely cover the grids on each coverslip.

Note: If needed, additional immunolabeling can be performed simultaneously, with other non-rabbit primary antibodies.

Note: We recommend assessing unspecific binding of secondary antibodies by skipping this step for one sample.

33. After incubation with the primary antibody, replace coverslips in wells of a 4-well plate containing PBS.
34. Gently wash 6 times for 5 min with 500 µL of PBS per well at room temperature.
35. Incubate sheets with 15 nm gold particle-conjugated goat anti-rabbit IgG (troubleshooting 3)
    a. Prepare a 1% solution of the secondary antibody in the blocking solution.
    b. Remove PBS from wells and add 400 µL of secondary antibody dilution per well.
    c. Incubate for 1 h at room temperature.

Note: If several primary antibodies have been used simultaneously, use secondary antibodies with different diameter of gold particles.

36. Remove secondary antibody solution and gently wash 6 times with 500 µL of PBS per well at room temperature.
37. Remove PBS from wells and incubate samples with 400 µL per well of 2.5% ice-cold glutaraldehyde solution for 2 h at 2°C–8°C.

Note: From this step, all the sample treatment should be done under a chemical hood.

38. Remove fixative from wells and rinse in sodium cacodylate buffer 0.1 M for 2 h at room temperature.
39. Postfix with 0.5% OsO_4 solution for 45 min - 1 h at room temperature.
40. Remove fixative from wells and rinse with sterile ultrapure water for the same time that osmium post-fixation (45 min - 1 h) at room temperature.
41. Dehydrate samples by incubating with a graded series of ethanol (25, 50, 70, 95 and 100%), each for 15 min.
42. Incubate samples with 400 µL per well of HMDS - ethanol 50%/50% solution for 15 min at room temperature.
43. Incubate samples with 400 µL per well of HMDS for 2 × 15 min at room temperature.
44. Air-dry samples overnight under the chemical hood.

Pause point: Samples can be stored at room temperature to be imaged later.

Image acquisition by TEM

Timing: 4–8 h (depending on the number of coverslips)

The purpose of this step is to obtain images of the localization of the PA probe on the cytosolic face of the plasma membrane by electron microscopy.

45. Gently take out one grid from the coverslip with a forceps.
46. Mount the grid in a standard TEM holder and insert the holder into the TEM.
47. Scan the grid at low magnification (× 25,000) to find stained sheets with docked secretory granules.
48. Acquire at least 30 images per condition at high magnification (× 60,000).

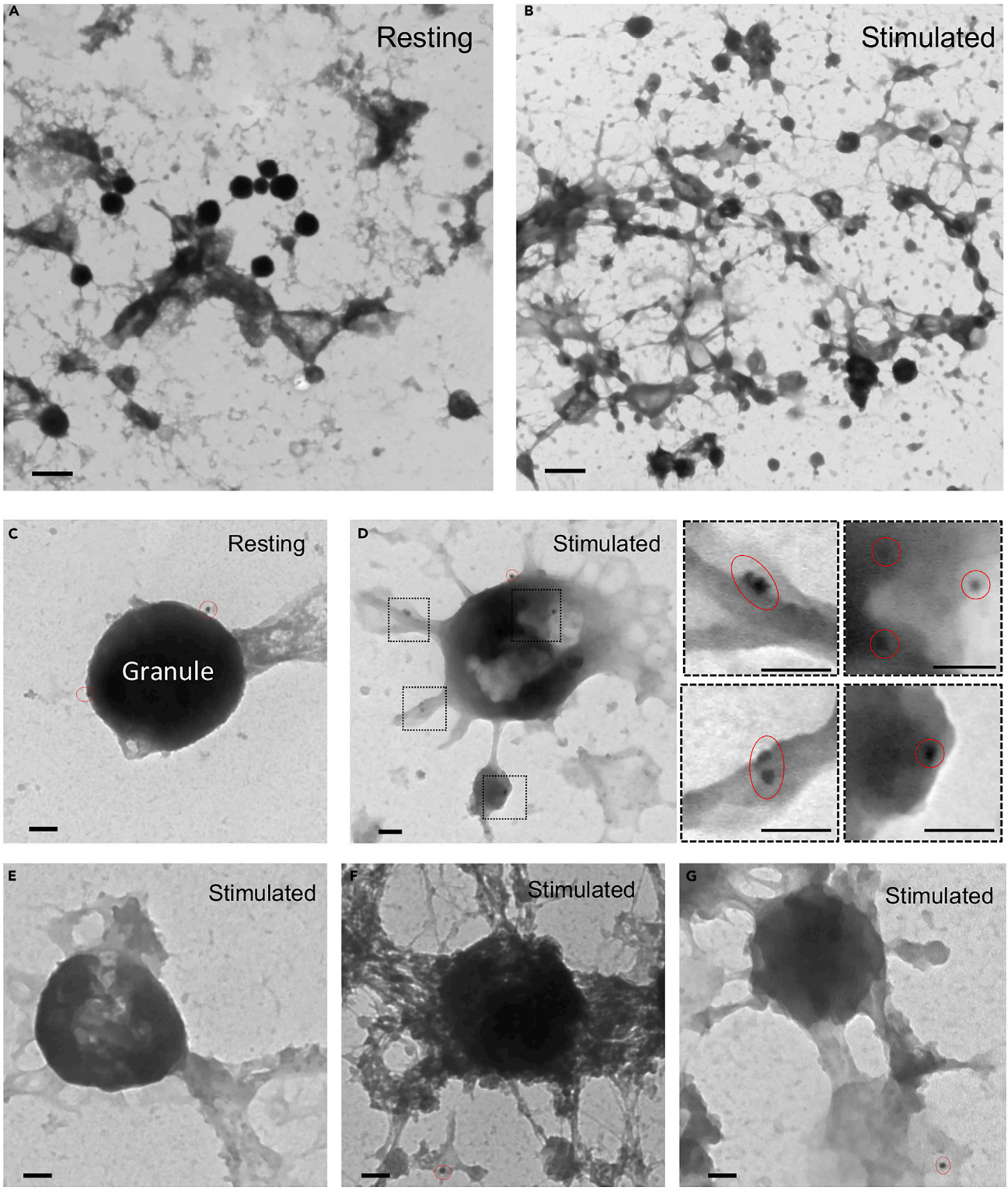

Figure 2. TEM imaging of plasma membrane sheets from chromaffin cells immunolabeled to reveal GFP distribution (gold particles highlighted by red circles).

(A and B) Low-magnification images of plasma membrane sheets from resting (A) or stimulated (B) cells expressing Spo20p-GFP (× 25 000, bars = 500 nm).

Figure 2. *Continued*

(C and D) High-magnification images of plasma membrane sheets from resting (C) or stimulated (D) cells expressing Spo20p-GFP (× 60 000, bars = 100 nm). Zooms (dotted squares) illustrate recruitment of PA sensor after stimulation of exocytosis, both below the granule and at granule anchoring structures near the exocytotic site.

(E–G) Higher magnification images of plasma membrane sheets illustrating controls for labeling specificity (× 60 000, bars = 100 nm). Immunolabeling was performed without primary GFP antibodies (E), in cells expressing only GFP without PA sensor (F), or in cells expressing PDE4A1-GFP, a PA probe unable to bind PA at the plasma membrane (G).

EXPECTED OUTCOMES

Between 16–18 h after electroporation, the number of adherent cells is around 250,000 per coverslip, due to the inherent high toxicity of electroporation technique (≈85% of cell death). The transfection yield of Spo20p-GFP plasmid is generally around 10%. Spo20p-GFP probe is mostly localized in the nucleus when cells are in resting conditions, of which a portion is recruited to the plasma membrane after stimulation of exocytosis (Kassas et al., 2012; Zeniou-Meyer et al., 2007).

On plasma membrane sheets from resting cells, the few docked dense-core granules appear round-shaped and smooth. However, on sheets from stimulated cells, granules are mostly fused and surrounded by a filamentous network (Figures 2A and 2B).

Despite evidence that PA plays an essential role in regulated exocytosis (Tanguy et al., 2016, 2018, 2019), its precise location at the exocytotic site remains unknown. According to previous studies showing a recruitment of Spo20p-GFP during exocytosis (Kassas et al., 2012; Zeniou-Meyer et al., 2007), TEM imaging of plasma membrane sheets revealed an increase in Spo20p-GFP near docked chromaffin granules after stimulation of exocytosis (Figures 2C and 2D). The PA sensor was mainly found associated with granule anchoring structures (Figure 2D). Beads were also found closely associated with fusing granules (becoming less dense after partial release of content). As these beads appear lower than the dense core by adjusting zoom and contrast during acquisition it is likely that they are found on the plasma membrane below the granule membrane (Figure 2D, see zooms on the right), but this could only by asserted with certainty by additional experiment approaches such as tomography.

The specificity of the PA labeling was assessed by comparing with other conditions such as non-transfected cells, mock-transfected, and cells incubated only with secondary antibodies. It was further tested in cells expressing GFP or PDE4A1-GFP, another PA probe which is mostly localized in the Golgi apparatus and is not recruited at the plasma membrane after stimulation of exocytosis (Carmon et al., 2020) or phagocytosis (Kassas et al., 2017). Under those conditions only background signal was detected in resting or stimulated condition (Figures 2E, 2F, and 2G), arguing that the labeling specifically reveals PA distribution.

To complete this protocol, it is also possible to observe plasma membrane sheets by electron tomography to obtain three dimensional images of exocytotic sites (Delavoie et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification analysis was done manually using the Adobe Photoshop software, but can also be done using free image analysis software such as ImageJ or Icy. The gold beads locations were determined by moving brightness and contrast using the "Levels" tool of the software (Figure 3A). For each condition, the numbers of beads per μm^2 were quantified using the "Count" tool to assess binding specificity and PA production near docking sites (Figure 3B). To decipher the distribution of the PA probe, the minimal distances between beads and granule border (from the bead towards the center of the granule) were quantified using the "Ruler" tool (Figure 3C). Data were collected in an Excel sheet (Figure 3D). This analysis was performed on at least 90 images per condition from three independent experiments. Statistical significance has been assessed by Mann-Whitney tests, using the SigmaPlot 13 software. Figure 4 illustrates the quantification of the number of beads per μm^2 in membrane sheets obtained from resting and K^+ stimulated cells expressing GFP, PDE4A1-

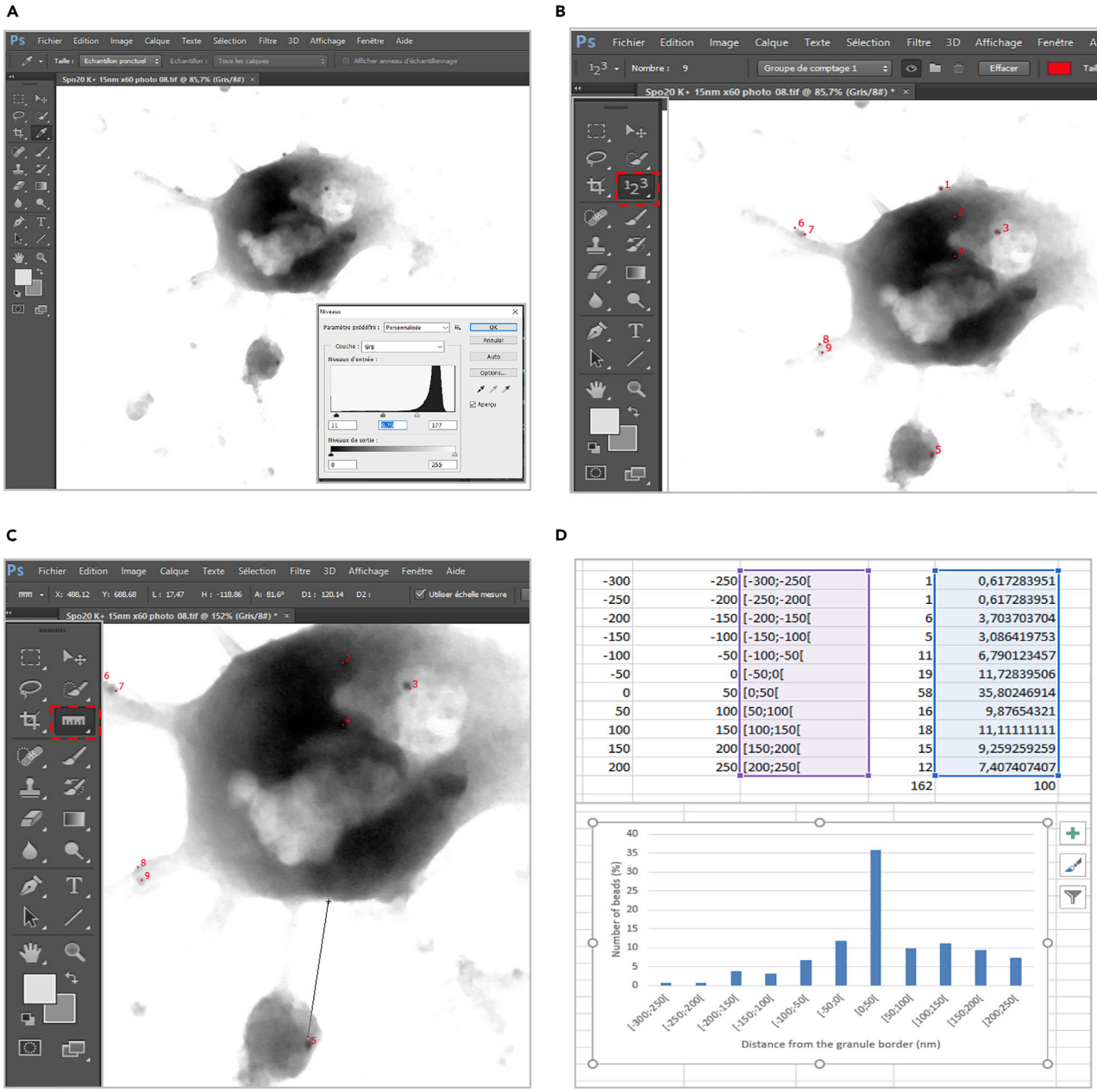

Figure 3. Quantification analysis of the immunogold GFP labeling

(A) Adjust brightness and contrast to reveal gold beads near a docked secretory granule using the "Levels" tool (shown in the inset).

(B) Number the beads in each field containing a docking site using the "Count" tool (shown into red dotted lines).

(C) Measure the distance between each bead and the granule border by using the "Ruler" tool (shown into red dotted lines). For beads below secretory granules a negative index was applied to the distance measured.

(D) Record each value in an Excel sheet. The distribution of the beads towards one docked granule can be plotted with the selected ranges.

GFP, or Spo20p-GFP. Note that when beads counting was restricted to areas within 250 nm of docked secretory granules the enrichment of beads in stimulated cells expressing Spo20p-GFP was even more obvious (see Tanguy et al., 2020).

These quantifications can be completed by assessing clustering and co-localization with other membrane lipids or proteins, using for instance Ripley's K-function (Gabel et al., 2015).

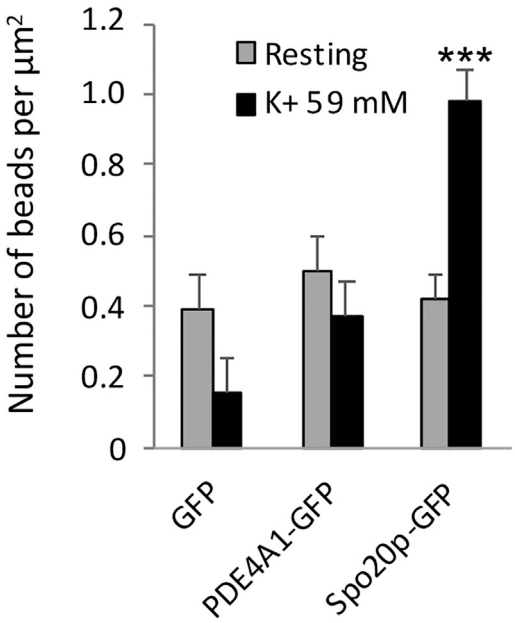

Figure 4. Secretagogue-evoked stimulation triggers the production of PA at the plasma membrane close to docked granules in chromaffin cells

The histograms show the mean number of gold beads per μm^2 from membrane sheets prepared from cells expressing GFP, PDE4A1-GFP, and Spo20p-GFP under resting or stimulated condition. Background signal in non-transfected cells was subtracted. Over 90 different membrane sheets were analyzed for each condition and the data presented as mean ± SEM. Statistical significance has been assessed by Mann-Whitney tests (*** $p<0.001$ compared to resting condition).

LIMITATIONS

This technique works optimally for adherent cells in monolayers and allows to observe docking of large dense core granules in secretory cells.

Transfection efficiency rarely exceeds 10%, which implies that a significant amount of time is spent to search stained sheets from the transfected cells during TEM observations.

Formvar films can present tiny air bubbles (only apparent during TEM imaging), even if grids are coated in a dry atmosphere. However, in our hands, this type of defect in Formvar films doesn't impact preparation of sheets and staining.

Like for all indirect imaging approaches one must keep in mind that the protocol described here allows for the detection of a PA sensor rather than for PA itself. Thus it is important to perform controls detailed above (Figures 2E, 2F, and 2G) to provide arguments for the specificity of the sensors and test for possible artifacts from sample preparation and immunolabeling.

TROUBLESHOOTING

Problem 1

Low transfection efficiency.

Potential solution

Try to optimize transfection conditions. The concentration, quality, and size of plasmid DNA can affect transfection efficiency, as well as the cell viability. A weak transfection yield can also be due to the presence of air bubbles during electroporation. Minimize formation of air bubbles and gently tap the cuvette before electroporation.

Problem 2

Plasma membrane sheets from cells in resting condition appear stimulated (high number of fused granules and/or high Spo20p-GFP recruitment on sheets).

Potential solution

Due to the high sensitivity of chromaffin cells to mechanical stimuli, resting cells may appear stimulated. Try to avoid any mechanical stress during medium changes. If nuclei are also present on sheets, try to apply less pressure on the cork when preparing sheets. If possible, prepare samples in duplicates.

Problem 3

High unspecific immunolabeling (background).

Potential solution

Ensure that it's not due to secondary antibody binding by performing immunostaining without primary antibody (anti-GFP). Reduce incubation time with secondary antibodies to 1 h maximum and extensively wash.

Unspecific antibody binding is also found after sample drying during staining or fungus development. Search for precipitates (crystals) or microbial contamination on sheets. Try to minimize sample drying by incubating in a humidity chamber and rapid immersion of samples. Use freshly prepared solutions to prevent contamination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nicolas Vitale (vitalen@unistra.fr).

Materials availability

This study did not generate new unique reagents/any new material.

The Spo20p-GFP expression vector used in this study is available upon request by contacting the lead contact.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

N.V. and S.G. conceived, designed, and supervised the project. E.T. conducted most of the experiments and data analysis. S.C.-G. developed the plasma membrane sheet technique and provided valuable comments for data analysis. C.R. and V.D. prepared Formvar-coated EM grids, treated samples, and performed TEM image acquisition. T.T. prepared fibronectin-coated coverslips, isolated bovine chromaffin cells, and prepared culture media. E.T. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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