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A Novel Protocol to Quantitatively Measure the Endocytic Trafficking of Amyloid Precursor Protein (APP) in Polarized Primary Neurons with Sub-cellular Resolution

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[Abstract] Alzheimer's disease's established primary trigger is β-amyloid (Aβ) (Mucke and Selkoe, 2012). The amyloid precursor protein (APP) endocytosis is required for Aβ generation at early endosomes (Rajendran and Annaert, 2012). APP retention at endosomes depends on its sorting for degradation in lysosomes (Haass *et al*., 1992; Morel *et al*., 2013; Edgar *et al*., 2015; Ubelmann *et al*., 2017). The following endocytosis assay has been optimized to assess the amyloid precursor protein (APP) endocytosis and degradation by live murine cortical primary neurons (Ubelmann *et al*., 2017).
Keywords: APP, Endocytosis, Degradation, Alzheimer's, Immunofluorescence

[Background] Aβ42 accumulation is a primary trigger of Alzheimer's disease. APP endocytosis is required for Aβ42 generation (Koo and Squazzo, 1994; Grbovic *et al*., 2003; Cirrito *et al*., 2008; Rajendran *et al*., 2008). The endocytosis of APP has been analysed in pulse-chase kinetic experiments in bulk by classical biotinylation of surface proteins (Sannerud *et al*., 2011; Xiao *et al*., 2012; Sullivan *et al*., 2014), in single cells by specific labelling of surface APP using antibodies against N-terminal extracellular domain of APP (Yamazaki *et al*., 1995; Xiao *et al*., 2012). The majority of these studies used non-neuronal cells (Yamazaki *et al*., 1996; Lee *et al*., 2008; Sullivan *et al*., 2014), and neuronal-like cell lines (Xiao *et al*., 2012), few used primary neurons (Yamazaki *et al*., 1995; Sullivan *et al*., 2014). Primary neurons differentiate like *in vivo* axons and dendrites, with their specialized presynaptic terminals and post-synaptic compartments. However, careful measurements and distinction between these neuronal compartments are lacking in these reports. We developed a method of analysing APP endocytosis in the different neuronal compartments, the soma or cell body, dendrites and axons that we describe in this bio-protocol. Our protocol details the procedure for following and measuring APP endocytosis in polarized neurons using classical immunofluorescence and semi-quantitative cell biology analysis methods.

We believe our method will allow the field to move forward by reliably measuring semi-quantitatively the compartmentalized endocytosis of APP specific to polarized neurons.

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Materials and Reagents

1. 24-well dishes (SARSTEDT, catalog number: 83.1836) for mammalian cell culture
2. Circular glass coverslips, 13 mm (VWR, Marienfeld, catalog number: 630-1597)
   Note: Autoclaved, pre-washed with 40% ethanol/60% HCl for 1 h at RT and washed 4 times, 15 min each, with Milli-Q water at RT; coated overnight with 200 µl 0.1% (w/v) poly-D-lysine at 37 °C in a 5% CO_2 and 20% O_2 humidified incubator and washed 3 x with sterile Milli-Q water.
3. Superfrost glass slides (MENZEL GERHARD, catalog number: 2586E)
4. Plastic Pasteur pipette (SARSTEDT, catalog number: 86.1171)
5. Parafilm (Fisher Scientific, catalog number: 11782644)
   Manufacturer: Bemis, Parafilm, catalog number: PM999.
6. Wild-type females and males mouse embryos (Balbc, embryonic day 16; Charles River)
7. APP-RFP plasmid (Szodorai *et al*., 2009) (S. Kins, University of Kaiserslautern)
8. 0.1% (w/v) poly-D-lysine (Sigma-Aldrich, catalog number: P1149)
9. Plating medium:
   a. DMEM, high glucose, pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11995065)
   b. 10% fetal bovine serum (FBS), qualified, heat inactivated, US origin (Thermo Fisher Scientific, Gibco™, catalog number: 16140071)
   c. 1% penicillin-streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
10. Neurobasal medium:
    a. Neurobasal medium (Thermo Fisher Scientific, Gibco™, catalog number: 21103049)
    b. 2% B-27® supplement, custom (50x) (Thermo Fisher Scientific, Gibco™, catalog number: 0080085SA)
    c. 0.1% GlutaMAX™ supplement (Thermo Fisher Scientific, Gibco™, catalog number: 35050038)
11. Trypsin (2.5%), no phenol red (Thermo Fisher Scientific, Gibco™, catalog number: 15090046)
12. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668019)
13. Opti-MEM (Thermo Fisher Scientific, catalog number: 31985062)
14. Murine anti-APP N-terminal monoclonal (22C11) (Merck, catalog number: MAB348)
15. HEPES (1 M) (Thermo Fisher Scientific, Gibco™, catalog number: 15630080)
16. Phosphate buffer saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10010031)
17. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148)
18. Sucrose (NZYTech, catalog number: MB18601)
19. Saponin (Sigma-Aldrich, catalog number: 47036)
20. Donkey anti-mouse Alexa 488 (Thermo Fisher Scientific, catalog number: A-21202)
21. Coverslip-Slide Mounting solution (FluoroMount-G) (SouthernBiotech, catalog number: 0100-01)

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22. DAPI (Sigma-Aldrich, catalog number: D9542)
23. HBSS (GE Healthcare, Hyclone™, catalog number: SH30031.03)
24. 50% glucose in sterile water (NZYTech, catalog number: MB16801)
25. Bovine serum albumin fraction V (BSA) (NZYTech, catalog number: MB04602)

Equipment

1. CO_2 incubator for primary cell culture (BINDER, model: CB 160)
2. Counting chamber (Belden, Hirschmann, catalog number: 8100103)
3. Epifluorescence upright microscope Z2 (Carl Zeiss, model: Axio Imager Z2) equipped a 60x NA-1.4 oil immersion objective and an AxioCam MRm CCD camera (Carl Zeiss)

Software

1. ImageJ software (free download from http://rsb.info.nih.gov/ij/)
2. GraphPad Prism 6 (https://www.graphpad.com/scientific-software/prism/)

Procedure

Note: The APP endocytosis assay is performed on neurons cultivated for 9 days in vitro (9 DIV).

A. Cell culture and transfection (Day 1, Day 6, Day 8)
   1. Day 1: Prepare primary cortical neurons from cortices and hippocampi from wild-type females and males mouse embryos (E16) as previously described (Almeida *et al*., 2005 and 2006; Ubelmann *et al*., 2017). Briefly, dissociated neurons are plated on 24-well plates containing poly-D-lysine coated glass coverslips (5-10 x 10^4 cells/cm^2) in plating medium (DIV 0). After 3 h or overnight, replace the plating medium with Neurobasal medium allowing only neurons to grow and differentiate until use. (Almeida *et al*., 2005 and 2006; Ubelmann *et al*., 2017)
   2. Day 8: For expression of APP-RFP cDNA, 8 DIV primary cortical neurons are transiently transfected with Lipofectamine 2000 according to manufacturer's protocol, using 0.5 µg cDNA: 0.5 µl Lipofectamine mix in 25 µl Opti-MEM per well of 24-well plate with 250 µl fresh antibiotics-free Neurobasal medium and incubated overnight until assaying APP endocytosis .
      Note: Expect on average 10 transfected healthy neurons at 8 DIV at stage 5 of differentiation (Dotti et al., 1988) and the neurites should not show bead-like structures that indicate degeneration. This efficiency can be achieved using freshly prepared cDNA with a regular midi-prep kit (we use a local NZYTECH brand).
   3. (Optional) Day 6: For knockdown analysis, primary neurons at 6 days *in vitro* (6 DIV) were transfected with siRNA using Lipofectamine RNAiMax according to manufacturer's protocol, after substituting culture media with fresh antibiotics-free Neurobasal medium.

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B. APP endocytosis assay of 9 DIV primary neurons expressing APP-RFP (Figure 1) by the following steps (Day 9)

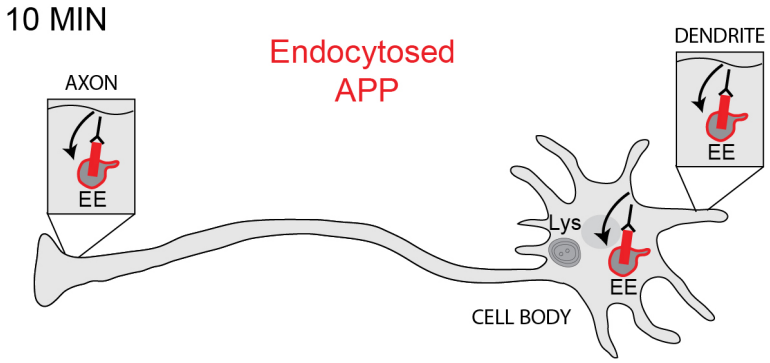

Figure 1. Schematic of APP endocytosis monitored using anti-APP antibody incubation for 10 min in axon, dendrite and cell body. EE: Early endosome; Lys: Lysosome.

1. Remove cell culture media with a plastic Pasteur pipette, add 400 µl B27-free Neurobasal medium for 30 min at 37 °C in cell culture incubator. All pipetting should be done slowly against the wall of the well without touching the cells.
2. Dilute 0.25 µl of anti-APP antibody (22C11; 0.25 µg/µl stock concentration) into 25 µl complete Neurobasal medium with 10 mM HEPES (0.25 µl of stock solution at 1 M, pH 7.2-7.5) per coverslip.
3. Place one 25 µl droplet of diluted anti-APP antibody per glass coverslip onto Parafilm stretched on a 24 wells plate lid.
4. Place the coverslips, carefully and as fast as possible, over the droplets with cells facing the antibody solution, cover the reaction in a container to avoid evaporation and incubate at 37 °C for 10 min in a cell culture incubator. This step allows for anti-APP antibody binding to cell surface APP and subsequent endocytosis.
   (Optional)
   a. To monitor endocytosed APP lysosomal degradation, further incubate cells for 60 min upon washing by dipping coverslips once for 4 s in 500 µl pre-warmed PBS.
   b. To detect APP at the plasma membrane, incubate cells with anti-APP antibody for only 4 min at 37 °C in a cell culture incubator. This optional step allows labelling anti-APP antibody bound to cell surface APP without significant detection of endocytosis.
5. Wash each coverslip by dipping once in 500 µl pre-warmed PBS for 4 s.
6. Fix cells by placing coverslips back in a 24-well plate and add 500 µl 4% paraformaldehyde/4% sucrose for 20 min at room temperature (RT). Replace fixative solution with 500 µl PBS, and after 3 washes proceed for detection of anti-APP.

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C. Anti-APP antibody detection (Day 9)

1. For detection of endocytosed APP bound to anti-APP antibody, permeabilize fixed cells with 500 µl 0.1% saponin/PBS (permeabilization buffer) for 60 min at RT. Remove the permeabilization buffer and wash each coverslip with PBS.

   Optional: For detection of anti-APP bound to APP at the cell surface (4 min), no permeabilization is required. **Do not include saponin in steps C2 and C3**.
2. Block non-specific binding of the antibody to serum proteins with 500 µl of blocking buffer (3% FBS/0.1% saponin/PBS) for 60 min at RT.
3. Place each coverslip (cells facing down) onto a 50 µl droplet of diluted donkey anti-mouse Alexa 488 (1:250) in 3% FBS/0.1% saponin/PBS.

   Optional: Include DAPI in the antibody solution (1:10,000 of a stock of 1 mg/ml) to counterstain cell nuclei.
4. Cover the reaction and incubate it for 60 min at RT, in the dark.
5. Place coverslips back on a 24-well plate and wash 3 times with PBS at RT.
6. Mount coverslips with cells facing down onto a 25 µl droplet of Fluoromount G at RT on microscope slides and let dry overnight in the dark to preserve the fluorescence signal.

   Note: Pipette Fluoromount G quickly to prevent it from drying but gently to avoid bubbles.

D. Image acquisition (Day 10)

Image acquisition is done using epifluorescence microscopy (such as ZEISS Z2) with a 60x NA 1.4 oil immersion objective and a CCD camera (see Equipment for details on microscope used). See Figure 2 for a representative image.

1. Neuronal cell body, portions of dendrites and axons must be in focus. Not well-developed neurons or neurons expressing low or high levels of APP-RFP should be excluded. Exposure times should be determined based on the sample with the brightest expected signal, to use as much of the dynamic range of the camera (ideally use a camera with 16-bit binary range) without saturating any of the pixels.
2. Acquire 10-20 neurons per condition to have sufficient data for statistical analysis. To image the whole neuron, it may be necessary to acquire multiple fields.

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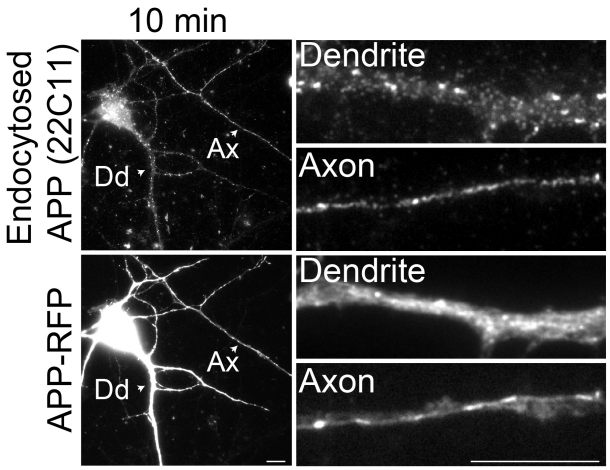

Figure 2. Representative image of APP endocytosis by primary neurons expressing APP-RFP incubated for 10 min with anti-APP (22C11) detected with anti-mouse Alexa 488. Dd: dendrite; Ax: axon; scale bars = 10 µm (adapted from Ubelmann *et al*., 2017).

E. Image analysis & APP endocytosis quantification in cell body, dendrite and axon (Figure 3) (Day 10-11)

Note: Quantification of endocytosed fluorescent signal acquired with ImageJ/Fiji.

1. Identify the cell body, a dendrite and the axon based on neuronal morphology and on RFP signal and roughly outline composite selections (non-contiguous ROIs) corresponding to cell body, two to five ~20 µm segments in dendrites and one in the axon (usually thinner, longer and with branches at 90° angle) using ‘polygon selection’ while pressing ‘shift’ (see Figure 3). Axons and dendrites can alternatively be counterstained with Ankyrin-G (Santa Cruz Biotechnology) or MAP2 (Sigma-Aldrich).

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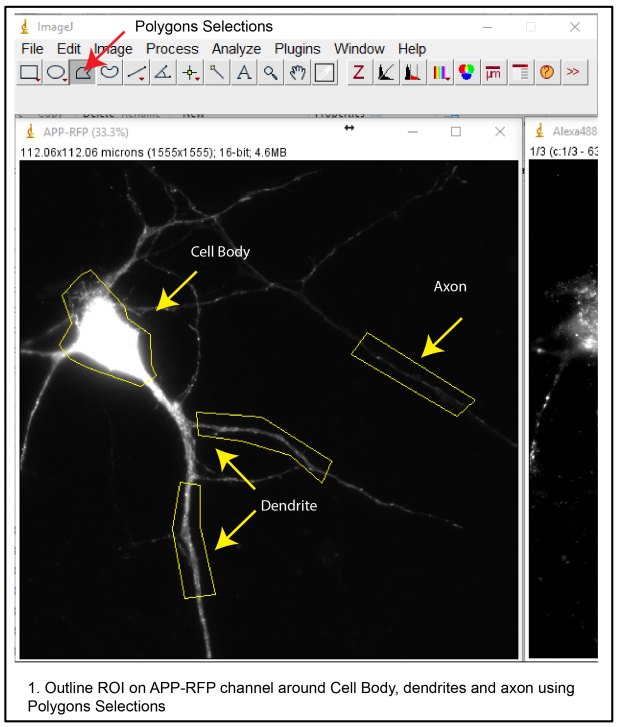

Figure 3. Image analysis of APP endocytosis: Step 1

2. Duplicate image (shift + D) containing composite selections and remove unselected pixels by using ‘Clear outside’ (see Figure 4).

Figure 4. Image analysis of APP endocytosis: Step 2

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3. To refine the ROI to the cell boundary: auto ‘Threshold’ the APP-RFP signal in the cell body; create a new ROI by clicking on the thresholded neurite with the ‘Magic Wand’; add ROI to ‘ROI manager’ (shift + t) (see Figure 5).

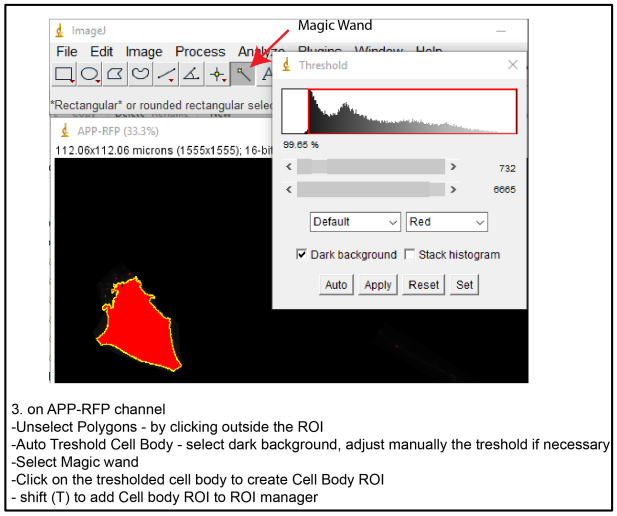

Figure 5. Image analysis of APP endocytosis: Step 3

4. To refine the ROI to the dendrites and axons: adjust the auto ‘Threshold’ to the APP-RFP signal in the dendrites and in the axon if necessary; create new ROIs by clicking on the thresholded neurites with the ‘Magic Wand’; add ROIs to ‘ROI manager’ (shift + t) (see Figure 6).

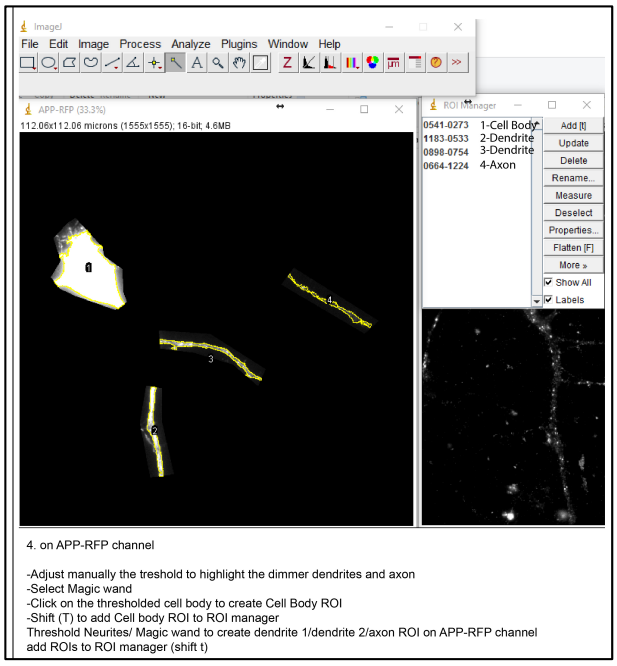

Figure 6. Image analysis of APP endocytosis: Step 4

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5. Transfer all regions to endocytosed Alexa488-anti-APP channel, draw a background ROI in a cell-free region using the tool ‘polygon selection’ and add it to the ‘ROI manager’. In the ROI manager, press ‘measure’ to obtain the mean Alexa488-anti-APP fluorescence intensities per ROI (background, cell body, dendrites and axon). Select, copy and export measurements to Microsoft Excel (see Figure 7).

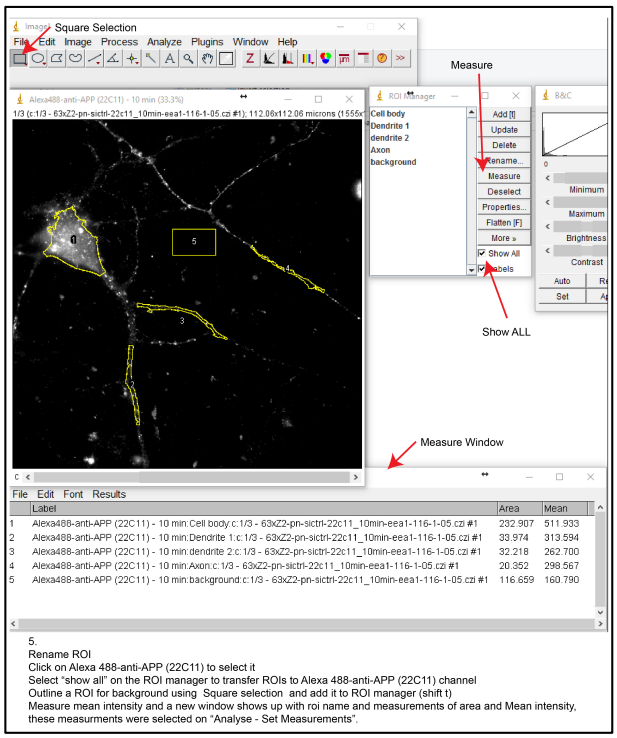

Figure 7. Image analysis of APP endocytosis: Step 5

6. Transfer all regions back to APP-RFP channel, and in the ROI manager, press ‘measure’ to obtain the mean APP-RFP fluorescence intensities per ROI (background, cell body, dendrites and axon). Select, copy and export measurements to Microsoft Excel (see Figure 8).

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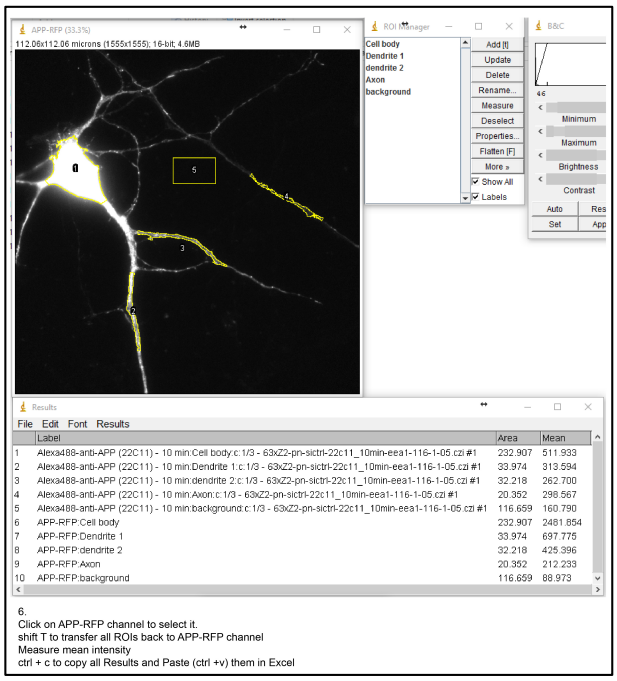

Figure 8. Image analysis of APP endocytosis: Step 6

Data analysis

Note: Data analysis is done using Microsoft Excel.

1. Subtract the background mean fluorescence intensity from all measurements, per single neuron.
2. Normalize the Alexa488-anti-APP fluorescence intensities (from the 3 neuronal compartments) by the APP-RFP fluorescence intensity in the cell body to control for the different expression level of APP-RFP, per single neuron.
3. Different conditions can be compared using a classical *t*-test provided the data follow a normal distribution.
4. The sample size is about 20 cells per condition in each independent experiment, based on previous studies. Statistical significance for at least three independent experiments is determined on normal data (D'Agostino-Pearson omnibus normality test) by two-tailed Student's *t*-test and for multiple comparisons one-way ANOVA with Tukey's test using GraphPad Prism 6.
5. Statistical significance for nonparametric data was tested by Mann-Whitney test.

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Notes

1. Variability is often due to the culture of primary neurons; the level of differentiation should be kept constant between independent experiments.
2. In our hands, APP endocytosis is robust with the average results very reproducible. However, it is expected variability between different neurons in the same experiment.
3. For optimal APP transfection and thus experimental conditions, good quality DNA plasmid and healthy and well developed primary neurons are paramount.

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