Chapter 3

Imaging Golgi Outposts in Fixed and Living Neurons

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Abstract

Here we describe the use of confocal microscopy in combination with antibodies specific to Golgi proteins to visualize dendritic Golgi outposts (GOPs) in cultured hippocampal pyramidal neurons. We also describe the use of spinning disk confocal microscopy, in combination with ectopically expressed glycosyltransferases fused to GFP variants, to visualize GOPs in living neurons.

Key words Neurons, Cultures, Dendrites, Golgi outposts, Confocal microscopy, Live imaging

1 Introduction

Cultures of embryonic rat hippocampal pyramidal neurons [1] have become a widely used model for analyzing the mechanisms underlying the development of axons and dendrites. Using this cell system it has been established that membrane trafficking is central for neuronal polarization [2, 3]. In fact, elements of the secretory pathway, such as the Golgi apparatus (GA) have a pivotal role in the establishment and maintenance of neuronal polarity and synaptic plasticity [4, 5]. In neurons, the GA not only consists of perinuclear cisternae but also of satellite tubule-vesicular structures designated as Golgi outposts (GOPs) that localize to dendrites [6–8]. GOPs have been implicated in dendritic morphogenesis and as stations for the local delivery of post-synaptic membrane receptors [5]. Here, we describe methods for the visualization of GOPs in fixed and living cultured hippocampal pyramidal neurons.

2 Materials

2.1 Neuron Isolation, Growth, and Plating

1. CMF-HBSS (Calcium-, magnesium-, and bicarbonate-free Hank's balanced salt solution (BSS) buffered with 10 mM HEPES, pH 7.3).

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2. Neuronal Plating Medium (MEM supplemented with glucose (0.6% wt/vol) and containing 10% (vol/vol)) horse serum.
3. Neurobasal/B27 Medium: Prepare according to the manufacturer's instructions by supplementing Neurobasal Medium (with or without Phenol red) (Gibco) with GlutaMAX-I CTS supplement (Gibco), B27 serum-free supplement (Gibco) and penicillin–streptomycin.
4. 2.5% (wt/vol) trypsin; aliquot for single use and store at –20 °C.
5. Poly-L-lysine (1 mg/ml) in 0.1 M borate buffer pH 8.5 and filter-sterilize (prepared immediately before use): Poly-L-Lysine, molecular weights 30,000–70,000 kDa; 0.1 M Borate buffer, pH 8.5 (prepared from boric acid and sodium tetraborate).
6. 5 mM AraC (Cytosine-1-β-D-arabinofuranoside); aliquot and store at 4 °C.
7. Lipofectamine 2000.
8. Coverslips Assistant #1 de 25 mm (Carolina Biological Supply Company).
9. 70% nitric acid (wt/wt).
10. Fire-polished glass Pasteur pipette.

2.2 Expression Plasmids

1. For neuronal live imaging [9] of the Golgi apparatus and dendritic GOPs we use several cDNA plasmid coding for the N-terminal domains (cytosolic tail, transmembrane domain, and a few amino acids of the stem region) of Golgi resident enzymes (e.g., glycosyltransferases; [10]) fused to the N terminus of the enhanced yellow fluorescent protein (YFP) or the red fluorescent protein, mCherry. The following constructs are routinely used for labeling the GA and GOPs in living or fixed cultured neurons: (1) Sialyl-transferase 2 (SialT2), a marker of the cis-Golgi; (2) Galactosyl-transferase 2 (GalT2), a marker of the media- and trans-GA; and (3) β-1, 4-*N*-acetylgalactosaminyltransferase (GalNAcT), a marker of the trans-GA. For further details [8, 10–12].

2.3 Antibodies

1. A mouse monoclonal antibody (mAb) against GM130, a marker of the cis-Golgi compartment (Clone 35/GM130; BD Biosciences) diluted 1:250.
2. A mAb against Mannosidase II, a marker of the Golgi apparatus (clone 53FC3, ab24565; AbCam), diluted 1:100.
3. A rabbit polyclonal antibody against TGN38, a marker of the TGN (Product Number T9826, Sigma Chemical Co.) diluted 1:250.
4. A mAb against MAP2 (clone AP20, M 1406, Sigma Chemical Co.) diluted 1:500.

5. Goat anti-mouse IgG-Alexa 488 conjugated (A-11001, Life Technologies).
6. Goat anti-rabbit IgG-Alexa 546 conjugated (A-11035, Life Technologies, Thermo Fisher Scientific Inc. Rockford, IL, USA).
7. Goat anti-mouse IgG-Alexa 633 conjugated (A-21050, Life Technologies, Thermo Fisher Scientific Inc. Rockford, IL, USA).
8. Goat anti-rabbit IgG-Alexa 633 conjugated (A-21070, Life Technologies, Thermo Fisher Scientific Inc. Rockford, IL, USA).

2.4 Immunofluorescence

1. Phosphate buffered saline (PBS), pH 7.2–7.4.
2. 4% paraformaldehyde (PFA), 4% sucrose in PBS buffer.
3. 0.2% Triton X-100 in PBS.
4. 5% bovine serum albumin (BSA) in PBS.
5. 1% BSA in PBS.
6. FluorSave™ Reagent (or similar medium) for mounting coverslips on microscope slides.

2.5 Microscopy

1. Conventional confocal microscopes: Zeiss Pascal or Olympus FV300.
2. Spectral confocal microscope: Olympus FV1000.
3. Spinning disk confocal microscope: Olympus IX81 plus Disk Spinning Unit (DSU).

3 Methods

3.1 Preparation of Coverslips

Cultured hippocampal neurons are grown attached to glass coverslips (12, 15, 25 mm in diameter) coated with different substrates (e.g., poly-lysine, laminin, and tenascin). Careful preparation of glass coverslips that serve as substrates for neuronal growth is a key step to assure appropriate development of axons and dendritic arbors. For most experiments we coated glass coverslips with poly-L-lysine (molecular weight 30,000–70,000).

1. Place 25-mm glass coverslips in porcelain racks in concentrated 70% nitric acid for 24 h (range 18–36 h).
2. Wash the coverslips (still in the racks) with distilled water three times/30 min each and then with Milli Q (type1 ultrapure) water two more times for 30 min with constant stirring.
3. Place the racks in a glass beaker covered with aluminum foil and sterilize in an oven at 220 °C for 4–6 h.

   Carry out all the following procedures sterile under a laminar flow hood.

4. Place coverslips in a 35-mm cell-culture dish or in a 6-multi well culture plate.
5. Cover each coverslip with the Poly-L-lysine solution (minimum 150 µl per coverslip). Leave at room temperature in the laminar flow hood (~20–25 °C) overnight.
6. Rinse the coated coverslips with sterile ultrapure MilliQ water every 20 min over a period of 2 h.
7. Discard water from the last wash and add Neuronal Plating Medium. Place the culture dishes in a CO_2 incubator at least 2 h before plating neurons. (Dishes can be stored in the incubator for several days before using them.)

3.2 Primary Cultures of Rat Hippocampal Neurons

1. Extract the brain from embryonic day 18 (E18) rat embryos and kept them in a dish with ice-cold Hank's.
2. Under a dissecting microscope, carefully remove the meninges from cerebral hemispheres and then dissect out the hippocampus. Collect the hippocampi in a dish containing ice-cold CMF-HBSS.
3. Place the extracted hippocampi in a 15 ml centrifugal tube and load up to 2.7 ml of CMF-HBSS. Add 0.3 ml of 2.5% trypsin and incubate for 15 min in a water bath at 37 °C.
4. Remove the trypsin solution, and rinse the hippocampi two times with 5 ml of CMF-HBSS.
5. Using a fire-polished glass Pasteur pipette, mechanically dissociate hippocampi pipetting them up and down (five to ten times). Then repeat this procedure with narrowed flame-polished pipette. At this point, there should be no chunks or tissue left.
6. Plate the desired number of cells (between 100,000 and 150,000) in the culture dishes containing the coated coverslips in the Neuronal Plating Medium and place them in the incubator at 37 °C with 5% CO_2 for 2–4 h.
7. Replace the Neuronal Plating Medium for warmed (37 °C) Neurobasal/B27 Medium and incubate at 37 °C with 5% CO_2.
8. After plating for 72 h, add AraC (Cytosine-1-β-D-arabinofuranoside) to a final concentration of 5 µM to inhibit proliferation of glial cells. To maintain the culture, every 3 days replace 1/3 medium with fresh Neurobasal/B27 Medium.

   For further details *see* ref. [1] (*see* **Note 1**).

3.3 Transient Transfection of Cultured Hippocampal Neurons

For visualization of GOPs in living and fixed neurons we transiently transfect 14 days in vitro (DIV) cultured hippocampal neurons (*see* **Note 2**) with cDNAs encoding one of several resident Golgi glycosyltransferases (see above) (*see* **Note 3**).

1. Under a laminar flow hood, collect and store the Neurobasal/B27 medium from the cultured neurons and replace it with warm 2 ml Neurobasal.
2. For one (1) 35-mm cell-culture dish:

   Add to one tube 62.5 μl Neurobasal + 2 μl Lipofectamine 2000.

   Add to another tube 62.5 μl Neurobasal + 0.5–1 μg DNA.
3. Incubate for 5 min at room temperature (RT).
4. Combine the contents of the tubes from **steps 2**- with gentle mixing.
5. Incubate for 20–25 min at RT.
6. Add the transfection mix to the cells and swirl gently.
7. Incubate for 1–2 h at 37 °C with 5 % CO_2.
8. Replace the transfection media with the original Neurobasal/B27 medium and return cells to the incubator.

3.4 Immunofluorescence of Fixed Cells

GOPs are also visualized in fixed cultures with specific antibodies to Golgi proteins. All steps for immunofluorescence labeling are performed at room temperature. For immunofluorescence of fixed cells, cultures are usually grown on 12-mm glass coverslips

1. *Fixation*. Rinse the cells with PBS at 37 °C. Remove PBS and immediately fix using 4 % PFA (paraformaldehyde)—4 % sucrose in PBS buffer at 37 °C, for 20 min. Paraformaldehyde is a popular fixative and will usually result in better preservation of cellular morphology than methanol or acetone.
2. Wash cells thoroughly (3–5 washes × 5 min in PBS).
3. *Permeabilize* with 0.2 % Triton TX-100 in PBS for 10 min.
4. Wash cells thoroughly (3–5 washes × 5 min in PBS).
5. *Block* by incubation with 5 % BSA (bovine serum albumin) in PBS for 1 h.
6. *Incubate with primary antibody*. Prepare primary antibody in 1 % BSA in PBS. Incubate for 1 h at room temperature or overnight at 4 °C.
7. Wash cells thoroughly (3–5 washes × 5 min in PBS).
8. *Incubate with secondary antibody*. Prepare secondary antibody (dilution 1:200) solution as in the case of primary antibodies. Incubate 1 h at room temperature. Longer incubation may increase background.
9. Wash cells thoroughly (3–5 washes × 5 min in PBS).
10. *Mounting*. We routinely use FluorSave™ Reagent.

3.5 Microscopy

3.5.1 Imaging Fixed Samples

To visualize fixed stained cells we acquired high resolution images (1024 × 1024 image size, 12-bit per pixel) using either a conventional (Zeiss Pascal) or a spectral (Olympus Fluoview 1000) inverted confocal microscope with an oil immersion PlanApo

60×/1.4 objective. We collect a 5–10 μm-deep Z stack along the optical axis to obtain voxel sizes of approximately $0.2 \times 0.2 \times 0.2$ μm. The image files were processed and analyzed with ImageJ software (*see* **Note 4**).

3.5.2 *Live Cell Imaging*

General imaging considerations: For time-lapse fluorescence microscopy, we used an Olympus IX81 inverted microscope equipped with a Disk Spinning Unit (DSU) and epi-fluorescence illumination (150W Xenon Lamp). We typically obtain 3–5 optical sections (700 nm each) every 2 s during a time period of 3–5 min. However, since dendrites are sometimes quite thin (1.0 μm thickness), the GA and GOPs can be completely captured and imaged with a single optical slice. In these cases, time-lapse sequences are acquired at a continuous rate of 2–5 frames per second during 3–5 min.

Objective

Cells plated on coverslips are well within the working distance of all microscope objectives, including high-NA PlanApo objectives, which offer the greatest optical correction and superior light gathering ability. These high-NA objectives are ideal for live cell fluorescence imaging. For instance, in our set up we use a 60× PlanApo 1.4 NA. In some cases, we have used TIRFM objectives with higher NA (1.45).

Cameras

Charge-coupled device (CCD) cameras have become the standard detectors for live cell imaging. CCD cameras provide high sensitivity and linear response over their dynamic range. Our DSU microscope set up is coupled with an ANDOR iXon3 CCD camera. We have also imaged GOPs using other cameras, such as the ORCA-AG (Hamamatsu) or the ORCA-ER (Hamamatsu).

Environmental Control

For recordings we use open chambers (Fig. 1), which allow cells to be maintained in bicarbonate-based media, inside a stage top incubator (INU series, TOKAI HIT) with 37 °C controlled temperature, 5% CO_2 and humidity (enriched atmosphere). In the chambers, the coverslips (25 mm diameter) with the attached cells rests face-up on a base plate. A rubber ring above the coverslip forms a well to hold the culture medium (Fig. 1). We have also used other devices such as a Harvard micro-incubator (Harvard Instruments, model PDMI-2). To avoid focus drift due to changes in objective temperature when contacting the sample we routinely use a lens heater collar.

Recording Media

We use medium without phenol red to avoid toxic breakdown products when exposed to light. For this, the cells are cultured in phenol red free-medium; alternatively, the cells can be grown in phenol red-free medium.

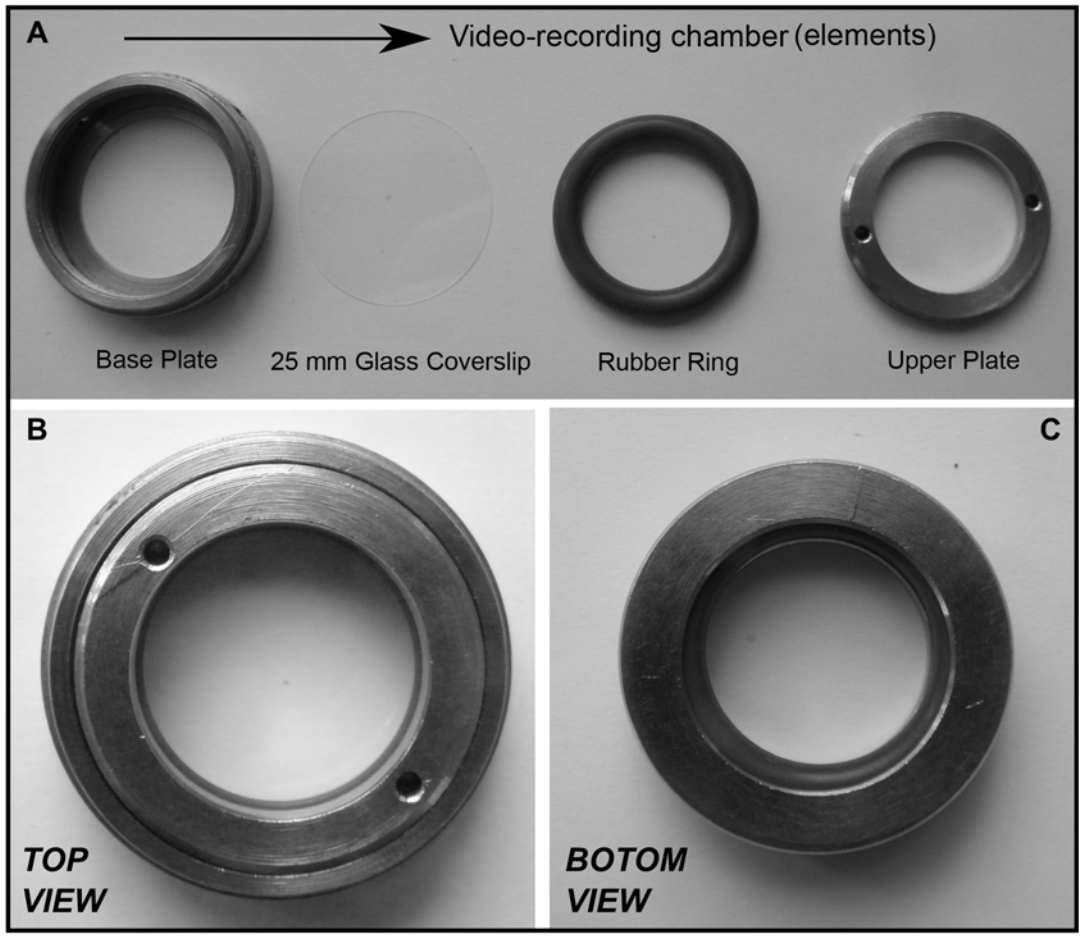

Fig. 1 (**a**) Components of the open chambers used for video recordings. They include: (a) base plate; (b) 25 mm coverslips, where the cells are grown; (c) a rubber ring, and (d) the upper plate. (**b**) Top view of the assembled chamber. (**c**) Bottom view of the assembled chamber

4 Notes

1. A major problem for the appropriate detection and visualization of GOPs is the quality of the neuronal culture. In our hands the best protocol for culturing embryonic hippocampal pyramidal neurons is the one developed and used in Gary Banker's lab [1].
2. Another important aspect for detection and visualization of GOPs is the age of the culture. GOPs are abundant in neurons with well-developed dendrites (long and highly branched MAP2 + neurites). These dendrites are found in neurons that have developed in culture for at least 10 DIV. We routinely use

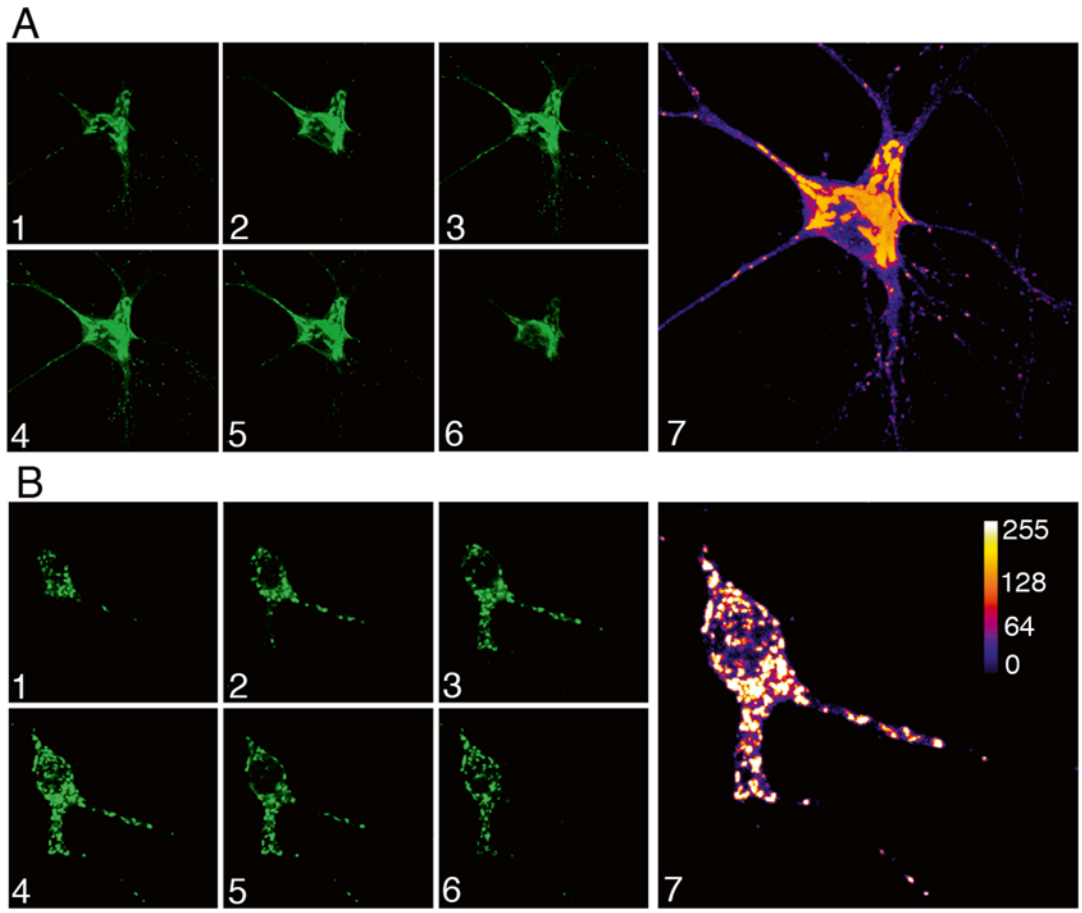

Fig. 2 (**a**) Serial (1–6) confocal micrographs showing the morphology of the GA and GOPs in a 14 DIV hippocampal pyramidal neuron transfected with SialT2-YFP (7). Pseudocolor (Fire LUT) maximal projection image obtained from the serial confocal micrographs shown previously. Note the presence of GA-derived tubules (*arrowheads*) (*large arrows*) and of small SialT2 + vesicular structures (GOPs, *arrows*) localized along dendrites. The neuron shows no signs of somatic GA fragmentation. (**b**) Another example of a 14 DIV neuron expressing SialT2-YFP. The Fire LUT (7) clearly reveals that this cell express much higher levels of ectopic protein than the one shown in panel (**a**). Neurons expressing high levels of ectopic resident Golgi glycosyltransferases quite frequently display somatic GA fragmentation and dispersal into dendrites. We routinely do not use neurons with fragmented GA

14-21 DIV neurons. GOPS are difficult to detect in young cultures (less than a week).

3. GOPS are easily visualized by ectopic expression of GA resident proteins, such as glycosyltransferases. However, when using this procedure care should be taken to avoid excessive expression of the ectopic protein. High expression levels of Golgi-resident enzymes can produce Golgi fragmentation and dispersal into dendrites (Fig. 2). Because of this, we only use neurons with moderate levels of expression and without signs of somatic Golgi fragmentation.

4. To highlight detection of small GOPs (less than 1 μm), either labeled by ectopic expression of fusion proteins or specific antibodies, we routinely used a look-up-table (LUT) called FIRE (scale thermal) available within the LUT table of FIJI ([13] *see* also Fig. 2).

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