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Detection of Microtubule Nucleation Hotspots at the Golgi

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Abstract

Cell polarization is important for multiple physiological processes. In motile cells, microtubules (MTs) are organized as a polarized array, which is to a large extent comprised of Golgi-derived MTs (GDMTs), which asymmetrically extend toward the cell front. We have recently found that GDMT asymmetry is based on a nonrandom positioning of spatially restricted nucleation hotspots, where MTs form in a cooperative manner. Here, we summarize methods used for GDMT identification including microtubule regrowth after complete drug-induced depolymerization and tracking of growing microtubules using fluorescent MT plus-end-tracking proteins (+TIPs) in living cells, and subsequent detection of those GDMTs that originate from the nucleation hotspots. These approaches can be used for quantification of the spatial distribution of MT nucleation events associated with the Golgi or another large structure.

Keywords

Microtubules; MTOC; Golgi; Nucleation; Hotspots

1 Introduction

The polarity of the MT network is important for directional migration and establishing polarity in a motile cell; both MT distribution and dynamics require asymmetry. To a large extent, MT asymmetry is achieved by MTs that nucleate from the Golgi apparatus [1, 2]. As opposed to radial centrosomal MT arrays, Golgi-derived MTs (GDMTs) comprise a major non-centrosomal asymmetric MT array. Our lab has shown that GDMTs enable directional persistence of cell migration serving as tracks for polarized trafficking and supporting organization of the Golgi itself [1–3]. These features likely underlie essential GDMT functions in neurons and motile and secretory cells [4–8].

While GDMTs bear essential cellular functions, it is still unclear what defines the distribution of MT nucleation sites at the Golgi, and what underlies GDMT asymmetry. We have recently shown that GDMTs are distributed at the Golgi surface in a nonrandom manner. Rather, they emerge from a small number of nucleation hotspots [9] (Figs. 1 and 2a). Interestingly, GDMT array polarity reflects the distribution of the hotspots, highlighting the significance of this specific nucleation location. Hotspots likely encompass clusters of highly concentrated active molecules ensuring efficient MT nucleation at the Golgi. Our results indicate that MTs within the hotspot are nucleated in a cooperative manner,

suggesting that recruitment and/or activation of molecular factors governing MT nucleation at the Golgi is locally facilitated by a preexisting GDMT.

MT nucleation from the centrosome has been studied by tracking fluorescently labeled +TIPs such as CLIP170 and EB1 [10, 11]. As +TIPs associate only with newly nucleated MTs and move away from the MT organizing center (MTOC), as a MT polymerizes, the emergence of one +TIP spot from the MTOC is considered as one nucleation event. Using this principle, we analyze MT nucleation at the Golgi by tracking Emerald-EB3-positive comets in interphase RPE1 or MRC-5 cells at steady state. A complementary approach is the complete disassembly of the existing MTs by nocodazole and observing the initiation of MT nucleation from the resulting fragments of the Golgi after nocodazole washout. In undifferentiated RPE1 cells, these approaches have previously allowed us to identify the Golgi apparatus as an alternative MTOC [4, 9]. Here, we use similar approaches to identify and further describe how to quantify microtubule nucleation hotspots at the Golgi.

2 Materials

2.1 Cell Culture and Transfection

1. RPE1 (Clontech) and MRC-5 (American Type Culture Collection) cells were maintained in DMEM/F12 with 10% FBS and MEM with 10% FBS (Lonza) media, respectively, and grown at 37 °C with 5% CO₂.
2. 0.25% Trypsin solution with EDTA, without sodium bicarbonate, calcium, and magnesium.
3. Ectopic expression of fluorescent markers achieved via transfection using Eugene 6 (Roche), or nucleofection (Amaxa).
4. Plasmids: As MT +TIP markers, Emerald-EB3 (kind gift from M. Davidson; Addgene plasmid # 54076) or another fluorescently tagged +TIP marker can be used. GFP-centrin (kind gift of Dr. M. Bornens, Paris; Addgene plasmid# 72641) can be used as a centrosome marker. To mark the Golgi apparatus, pmCherry-N1-GalT (kind gift from Lei Lu, Addgene plasmid# 83727), TGN-RFP [12], or GM130-GFP (kind gift from C. Sütterlin, University of California, Irvine [13]) can be used.

2.2 Live-Cell Imaging and Immunofluorescence

1. Cell culture dishes for live-cell imaging: 35 mm Glass-bottom dish (e.g., MatTek Corporation) with No. 1.5 coverslips.
2. Human fibronectin is dissolved in 1 M urea up to 2.27 µM (1 mg/mL) stock concentration.
3. Phosphate-buffered saline (PBS): Prepare and autoclave 10× stock solution: 1.37 M NaCl, 27 mM KCl, 80 mM Na₂HPO₄, and 20 mM KH₂PO₄, store at room temperature. The stock solution is diluted into 1× working solution before use.
4. Fixative: Methanol stored at −20 °C in the presence of 3 Å molecular sieves (Sigma) to remove water.

5. Nocodazole was prepared at 16.6 mM in DMSO (cell culture grade) and stored at -20°C .
6. Extraction buffer: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl_2 , 0.1% saponin, adjust pH to 6.9. Store at 4°C [14].
7. Blocking and antibody dilution buffer: 1% Bovine serum albumin (BSA)/PBS supplemented with 5% donor horse serum (DHS).
8. Primary antibodies: Mouse anti- α -tubulin (1:1000, DM1A; Sigma-Aldrich), mouse anti-GM130 (1:300; BD Transduction Laboratories), rabbit anti-Giantin (1:1000; Abcam), rabbit anti- β -tubulin (1:1000, Abcam), sheep anti-TGN46 (1:5000; AbD Serotec; now BioRad).
9. Secondary antibodies: Alexa488-, Alexa568-, and Alexa647-conjugated highly cross-absorbed secondary antibodies (Invitrogen), CF405M-conjugated highly cross-absorbed secondary antibodies (Biotium).
10. Coverslips are mounted in Vectashield Mounting Medium (Vector Labs) and can be stored at -20°C .

2.3 Confocal Microscopy

1. Laser scanning confocal microscope: Nikon A1r with advanced photokinetic capacity, based on a TiE Motorized Inverted Microscope with 405, 488, 568, and 647 nm excitation lasers; $\times 20$, $\times 40$, $\times 60$, and $\times 100$ lenses; and DIC module, equipped with a high-speed resonant scanner (30 fps), run by NIS Elements C software.
2. A 4-color spinning disk Yokogawa CSU-X1 confocal based on a Nikon Eclipse Ti-E inverted microscope with 405, 488, 568, and 647 nm excitation lasers, Photometrics 95B Prime Back-illuminated sCMOS camera for high-resolution fast multicolor 3D live-cell imaging, run by NIS Elements C software. Apochromat lens $\times 100$ NA 1.49 was used.
3. Tokai Hit Incubation Package 1 STXG incubation system for small dishes; 35 mm, 50/60 mm, chamber slide, chambered cover glass and slide only for Ti/Ti2 Piezo Z stage. This system includes all dish holder and dish fixing as standard. It includes temperature controller with built-in digital gas mixer for 100% CO_2 use.
4. Software packages such as Nikon Elements Software, Imaris (Bitplane), and Fiji (NIH) can be used for image analysis.

2.4 Super-Resolution Microscopy

For quantitative analysis of hotspots, structural illumination microscopy should be used. For example, images can be acquired using either DeltaVision OMX (GE Technology) or N-SIM (Nikon) systems.

1. DeltaVision OMX with 405, 488, 568, and 642 nm lasers, $\times 60$ Plan-Apo NA1.42 lens, and a CMOS camera.

2. Nikon N-SIM with 405, 488, 561, and 647 nm lasers, an SR Apo TIRF $\times 100$ NA1.49 lens, and an EMCCD camera (DU-987, Andor Technology).

2.5 Calculation of Distance Between GDMT Minus Ends

GDMT minus-end distances can be calculated using Excel, Matlab, or any other analysis software. Distance D between GDMT minus ends is calculated in 3D by using the distance formula:

$$D_{(a,b)} = \sqrt{((x_a - x_b)^2 + (r_a - r_b)^2 + (z_a - z_b)^2)}$$

3 Methods

GDMT nucleation hotspots are defined as spheres with a diameter of 0.4 μm containing both Golgi membranes and multiple ($n > 1$) MT minus ends. For hotspot analysis, it is necessary to determine positions of the minus ends of emerging MTs and the Golgi surface.

Three methods can be used to identify GDMT nucleation sites; all methods are briefly described below; for detailed methods refer to [15].

The first method involves transfection of cells with a +TIP marker and a Golgi marker followed by live-cell imaging to record MT tip movement in steady state (Fig. 2a). This method allows identification of nucleation sites in cells with unchanged MT organization. Imaging those cells in the appropriate cell cycle stage where the Golgi and centrosome are physically separated will help to clearly distinguish between centrosomal MTs and GDMTs and further accurately measure GDMT hotspot nucleation.

An alternative and more definitive method involves monitoring MT re-polymerization after nocodazole washout. When MTs are completely depolymerized upon nocodazole treatment, the Golgi subsequently fragments into ministacks which are distributed all over the cell. Imaging cells shortly after nocodazole washout can distinctly identify those MTs which are nucleated from the Golgi. The second method thus involves live imaging of cells expressing a +TIP marker and a Golgi marker in the course of a nocodazole washout (Fig. 3a, b).

For the purpose of statistical evaluation of MT number organized at the Golgi, a suggested third method involves nocodazole washout experiments in non-transfected cells with fixation at specific time points and immunostaining to visualize MTs and the Golgi.

3.1 Live-Cell Imaging of MT Nucleation in Steady State

1. Cell maintenance and preparation for transfection: RPE1 and MRC-5 cells are transfected on 35 mm tissue culture dishes at 80% confluency.
2. Transfection: Cells are transfected overnight using Fugene 6 or nucleofection according to the manufacturer's instructions. For live-cell imaging of steady-state MT dynamics, cells can be transfected with Emerald-EB3 and TGN-RFP (RPE1) or EB3-mCherry and GTN-GFP (MRC-5) (see Note 1). Post-transfection, cells are plated and grown on 35 mm glass-bottom dishes that have been coated with

fibronectin (1:100 dilutions in PBS, 30-min incubation at room temperature) and used for imaging.

3. Image stacks with 0.3 μm between slices can be recorded to cover the entire Golgi (over 3–4 μm) for 5 min with maximal frame rate (~4–5 s per stack) using the spinning disk confocal microscope (Fig. 2a)

3.2 Live-Cell Imaging of MT Nucleation After Nocodazole Washout

1. Cells are maintained and transfected as described in the above procedure (*see steps 1 and 2* in Subheading 3.1).
2. Nocodazole treatment: To disassemble MTs, the nocodazole stock is diluted with complete medium at 1:2000 dilution, added to the cells, and incubated at 37 °C for 2 h.
3. Nocodazole washout: Cells incubated with nocodazole are directly placed on the microscope stage for imaging. Cells are briefly imaged prior to nocodazole washout to obtain accurate pre-washout images (evidence of whole MT network disruption).
4. Cells are washed at least ten times with ice-cold medium at the stage, to remove all traces of nocodazole (*see Note 2*).
5. For live-cell imaging of nocodazole washouts, image stacks with 5 s intervals are taken using the spinning disk confocal microscope (Fig. 3a, b) for at least 10 min.

3.3 Immunofluorescence Analysis of MT Nucleation After Nocodazole Washout

1. Cells are maintained as mentioned previously (*see step 1* in Subheading 3.1).
2. Cells are plated and grown on fibronectin-coated coverslips (*see above*) (*see Note 3*).
3. Nocodazole treatment: Cells are treated with nocodazole as previously described (*see step 2*, Subheading 3.2).
4. Nocodazole washout for immunofluorescence: Two 60 mm tissue culture dishes containing 5 mL complete medium and extraction buffer are placed on a floating raft in a 37 °C water bath. To remove all traces of nocodazole while preventing MT repolymerization during washing, nocodazole-treated cells are placed directly on ice and the cells are washed with ice-cold plain medium for minimum ten times. Coverslips are quickly immersed in the 60 mm dish containing complete medium with gentle swirling of the dish for either 30, 45, or 60 s to allow MT regrowth. The coverslips are then immersed into extraction buffer for 15 s with gentle swirling to extract free tubulin from the cytoplasm. Cells are then subjected to fixation.
5. Cells are fixed with ice-cold 100% methanol at –20 °C for 10 min; coverslips are triple washed with PBS and allowed to rehydrate prior to blocking for at least 1 h at room temperature or overnight at 4 °C.

6. Cells are blocked with DHS/BSA for 1 h at room temperature.
7. Post-blocking, cells are incubated with primary antibody diluted in blocking buffer solution overnight at 4 °C.
8. Post-incubation with primary antibody, cells are triple washed with 1× PBS for 5 min, and incubated in secondary antibody diluted in blocking buffer solution for 1 h at room temperature.
9. Post-washing with PBS, coverslips are mounted on slides using Vectashield, sealed with nail polish, and dried and are then ready to be imaged. To retain fluorescence signal long-term, slides can be stored at −20 °C.
10. Fixed preparations are imaged by SIM microscopy for the best resolution outcome.

3.4 Detection of MT Nucleation-Site Positions

3.4.1 Steady-State Live Imaging

1. ImageJ (NIH), Imaris (Bitplane), or any other appropriate software package can be used for processing. Image stack sequences covering the entire Golgi during 3 min are used. To subtract background, time-lapse images are processed by rolling ball background correction. Brightness, contrast, and gamma settings are then adjusted to make both the major and minor structures visible.
2. All MT plus tip tracks are determined in the Z- and T-dimensions. Newly formed Emerald-EB3 comets (growing MT plus ends) in 3D can be tracked using the ImageJ plug-in MtrackJ [16] or Imaris particle tracking. To quantify only newly nucleated MTs throughout the movie, tracks starting in the bottom or top slices of a stack or in the first time frame are excluded. Similarly, all the plus tip signals are tracked through Z-planes to determine the true origin. The plus tip signals existing in the upper or lowest plane of the stack are not considered as the sites of origin because those MTs might be crossing the stack in Z-direction. All the other tracks initiated during the imaging have to be tracked back to the sites of origin.
3. The starting points of MT tracks are colocalized with the Golgi marker and roughly assigned as GDMTs or non-GDMTs.
4. A short (25 s–3 min) video starting at the first detection timepoint of each emerging GDMT is used to produce a maximum-intensity projection of GDMT.

3.4.2 Live Imaging of Nocodazole Washouts

1. In nocodazole washout video sequences, background subtraction and manual 3D tracking of emerging MTs can be utilized as in steady-state experiment above, and the putative GDMT subset identified.
2. A short (2–3 min) video starting at MT regrowth onset is used to produce maximum-intensity projections of GDMTs (Fig. 3a, b).

3.4.3 Fixed Immunostained Preparations After Short-Term Nocodazole

Washout—3D stacks acquired by SIM imaging are used for fixed-cell nocodazole washout analysis. For approximate detection of GDMT subset, MT ends are manually colocalized with the Golgi.

3.4.4 Precise Detection of GDMT Minus-End Position

1. Z-stacks produced via time projections of live imaging (*see above*) or fixed cells are used.
2. In each Z-plane where GDMT minus end is detectable, a straight-line scan plot is made using ImageJ. The line scans are used to find the intensity maxima at the MT minus end (the PSF center) and compared between Z-planes to find the outmost minus-end position in Z. Resulting X, Y, and Z coordinates of each GDMT minus end are recorded for further analysis.

3.5 Analysis of the Golgi Surface and Golgi Fragment Size

The Golgi is detected by a fluorescently labeled or immunostained Golgi marker (*see above*) (*see Note 4*).

1. The Golgi surface can be detected using the surface mapping function in Imaris (Bitplane).
2. In nocodazole washout experiments, the Golgi fragment size can be determined using ImageJ (Fiji by Ferret's diameter (XY) and minimum and maximum range (Z), resulting in center of mass coordinates (XYZ) and the radius of the fragment, which can then be used to determine the Golgi surface coordinates) (*see Note 5*).

3.6 Detection of the Analyzed Golgi Surface and Golgi Fragment Size

GDMT hotspots are quantified to contain the Golgi surface and at least two MT minus ends within 0.4 μm to each other.

1. The distance between each GDMT minus end and the Golgi surface is calculated using nearest neighbor algorithm in Matlab. Only MTs that are within 0.4 μm of the Golgi marker are considered true GDMTs.
2. The distances between all true GDMT minus ends are calculated. This can be performed in Excel or Matlab or using any other suitable software. A histogram is generated to evaluate the distribution of distances, and those within 0.4 μm to each other are demarcated as hotspot GDMTs (Figs. 2b and 3c).
3. Histograms are compared to a histogram of randomly distributed points within 0.4 μm of the Golgi fragment surface (Fig. 3c).

4 Notes

1. It is critical to select cells with appropriate expression levels, e.g., when overexpressed, +TIPs decorate the entire MT network which can affect MT

dynamics. Conversely, weak fluorescence signal requires high laser intensity and/or long exposure time, which can induce photodamage to the cells.

2. Use a pump for efficient aspiration (avoid touching the chamber with the pipet tip!). Alternatively, a large-volume flow chamber can be used. Image recording is resumed immediately after washing.
3. For each experimental condition, consider using a minimum of three coverslips.
4. To detect the Golgi surface in fixed preparations, it is recommended to use Giantin as a Golgi marker. Giantin is a transmembrane protein (recognizes aa 1–469, cytoplasmic side) which presents a “defined” Golgi signal lending itself well to surface mapping.
5. For analysis, the entire image file can be used; however cropping images is helpful to keep track of Golgi fragments throughout analysis process.

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Fig. 1.
Example of hotspots with GDMTs in MRC-5 cells 40 s after nocodazole washout. SIM image maximum-intensity projections. Tubulin, green. Giantin, magenta (Reproduced from [9] with permission from [MBoC])

Fig. 2.

Microtubules are nucleated at specific sites within the Golgi ribbon. **(a)** An RPE1 cell expressing Emerald-EB3 (green, MT +TIP marker) and TGN-RFP (magenta, Golgi marker). A maximum-intensity projection of a confocal spinning disk microscopy sequence over a 3-min period and Z thickness 3 μm is shown (**a**, **a'**). Inset in **a** is enlarged in **a'**, showing newly formed GDMTs formed at the same site (arrows); asterisk indicates centrosome position. **(b)** Quantification of nearest-neighbor distance between GDMT nucleation sites, based on 3D live-time imaging (Reproduced from [9] with permission from [MBoC])

Fig. 3. GDMT nucleation is spatially restricted to distinct hotspots. **(a)** RPE1 cell expressing EB3-GFP (green) and mCherry-GalT (red, Golgi marker) 4 min after nocodazole washout. Single-plane confocal spinning disk microscopy. **(b, b')** Example of simultaneous multiple GDMT nucleation events (arrows) at Golgi fragments following nocodazole washout. Frames from a time-lapse image sequence. **(b)** EB3-GFP, inverted grayscale image. **(b')** EB3-GFP (green) and mCherry-GalT (red, Golgi marker). Time from the start of the movie, minutes: seconds. **(c)** Newly formed GDMTs are distributed nonrandomly on Golgi fragments following nocodazole washout. Nearest-neighbor distances of GDMT minus ends were calculated for each fragment associated with multiple GDMTs (Reproduced from [9] with permission from [MBoC])