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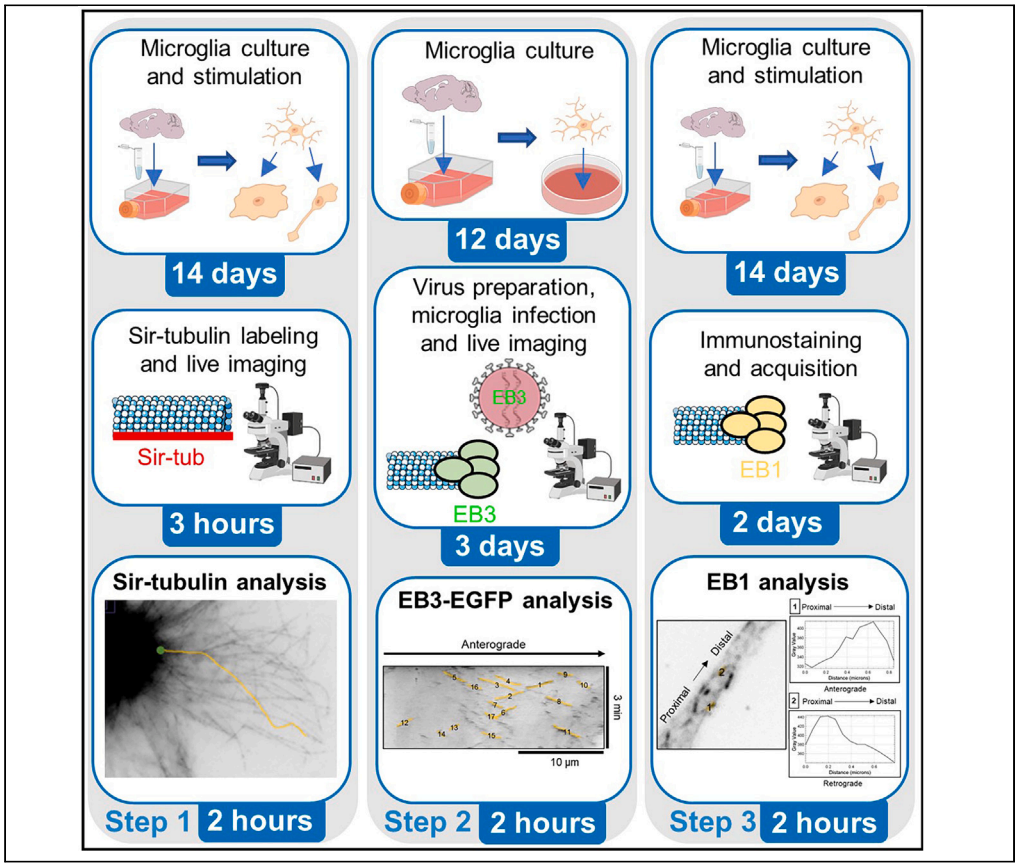
Protocol

Protocol for observing microtubules and microtubule ends in both fixed and live primary microglia cells



Microtubule dynamics and orientation have crucial roles in many vital cellular processes. However, functional live imaging of microtubules and/or microtubule ends in primary microglia can be challenging. Here, we present a protocol for observing microtubules and microtubule ends in both fixed and live primary microglia cells. We describe steps for microglia culture and *in vitro* stimulation, SiR-tubulin labeling, lentivirus preparation, live imaging, immunostaining, and image acquisition. We also provide procedures for SiR-tubulin, EB3-EGFP, and EB1 analyses.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Analysis of microtubule dynamics in primary homeostatic and reactive microglia

Analysis of microtubule plus end dynamics and orientation in homeostatic microglia

EB fluorescence gradients: a tool to determine microtubule orientation in fixed cells

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Protocol for observing microtubules and microtubule ends in both fixed and live primary microglia cells

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SUMMARY

Microtubule dynamics and orientation have crucial roles in many vital cellular processes. However, functional live imaging of microtubules and/or microtubule ends in primary microglia can be challenging. Here, we present a protocol for observing microtubules and microtubule ends in both fixed and live primary microglia cells. We describe steps for microglia culture and *in vitro* stimulation, SiR-tubulin labeling, lentivirus preparation, live imaging, immunostaining, and image acquisition. We also provide procedures for SiR-tubulin, EB3-EGFP, and EB1 analyses.

For complete details on the use and execution of this protocol, please refer to Rosito et al. (2023).[1]

BEFORE YOU BEGIN

The protocol below describes the step-by-step procedure to obtain primary murine microglia as described in Rosito et al.[1,2] Microglia are the brain's innate immune cells, which play a crucial role in maintaining a healthy brain environment. In their homeostatic state, microglia have a ramified morphology and continuously patrol the local environment by extension and retraction of highly motile processes.[3] However, in situations involving neuronal inflammation, injury, and neurodegenerative disorders, microglia undergo significant changes in both gene expression and morphology.[4–6] Despite the advantage of a controlled setting that primary cultures offer for investigating these cells, microglia *in vitro* experience widespread molecular alterations[7] as a result of the loss of signaling with other cells in the brain microenvironment, which is crucial for maintaining them in a state of homeostasis.

Hence, it is essential to fine-tune the culture conditions of these cells to minimize the modifications caused by the culturing environment. Here, we suggest a strategy that involves firstly a co-culture of astrocytes and microglia, than microglia isolation and plating with a medium with low serum concentration supplemented with astrocyte conditioned medium.[9] Since in the tissue microglia typically occupy distinct domains,[11,13] we also suggest plating cells at low cell density to avoid domain overlapping. Increased numbers of amoeboid-shaped microglia cells and/or decrease in the percentage of ramified forms are indications of microglia activation induced by culture conditions that is useful to consider throughout this entire protocol. For a detailed description of morphological parameters useful to discriminate activated morphologies, refer to Figure 1B-D of Rosito et al., 2023.[1]

It is possible to steer microglia toward different reactivity states,[15] such as an activated or an alternatively activated state, challenging the cells with either lipopolysaccharide-interferon γ (LPS-IFNγ) or interleukin-4 (IL-4), respectively. The details for this induced *in vitro* polarization are provided in *Preparation one*.

Prior to beginning these experiments, key resources (listed in the key resources table) should be available.

Before starting the dissection as described in *Preparation one*, coat T75 flasks with poly-L-lysine solution (5–10 mL in each flask) and expose them to UV light for 20 min. Prior to plating cells, rinse the flasks once with DMEM. This procedure can also be applied to multiwell plates, glass coverslips, or chambered slides (e.g., μ-Slide 8 Well, ibidi).

Prior to initiating the lentiviral preparation as outlined in *Preparation three*, plate HEK 293T cells on 150 mm dishes to achieve 60%–80% confluency on the day of transfection.

Institutional permissions

All procedures performed in this study using laboratory animals were in accordance with the Italian and European guidelines and were approved by the Italian Ministry of Health in accordance with the guidelines on the ethical use of animals from the European Communities Council Directive of September 20, 2010 (2010/63/UE). It is crucial to obtain the required permissions from the relevant institutions before carrying out the dissection steps outlined in this protocol. All experiments performed at Columbia University were approved by the Committee on the Ethics of Animal Experiments of Columbia University and performed according to Guide for the Care and Use of Laboratory Animals distributed by the National Institutes of Health.

Preparation one: Establishing the primary microglia cell culture

Timing: 14 days

Timing: 1 h (for step 1)

Timing: 1 h (for step 2)

Timing: 10–14 days, hands-on: 2 h (for step 3)

Timing: 48 h (for step 4)

1. Brain dissection: cortex isolation.
   a. Decapitate the mouse pup quickly with scissors. Pups should be anesthetized on ice accordingly to approved animal protocols and institutional permissions.
   b. Open the skull by gently cutting the skin and the skull in the median scissure, from caudal to rostral.
   c. Firmly grab the skin and the skull from the rostral part of the cut and tear it.
      i. Repeat for the second hemisphere.
      ii. Delicately but firmly pulling the tissues should be sufficient to remove both the skull and the skin.

   Note: the skull at this mouse age is really soft. Perform this procedure delicately to avoid damaging the brain tissue.

   d. Once exposed, transfer the brain into a petri dish on ice.
   e. Separate the cerebellum and the olfactory bulb from the rest of the brain.
   f. Separate the hemispheres.

g. Remove the meninges.
h. Separate the cortex from the subcortical structures.

Note: use a stereoscope to better visualize the subcortical structures.

i. Put the cortex in ice cold dissection medium in a 50 mL Falcon conical tube (maximum 5 cortices per tube) until the next step.

⚠ CRITICAL: the age of the pup will determine the yield of the cell culture. Use pups in the range P0–P1 for optimal yield, do not use pups older than P2. The yield is variable but optimally in the range of 1–2.5 x 10^6 cells per T75 flask.

2. Tissue digestion and cell plating.
   a. Aspirate dissection medium from the tube and wash twice with fresh dissection medium (see composition in the materials and equipment section).
   b. Aspirate the dissection medium and add 1.5 mL of papain solution *per* brain (see composition in the materials and equipment section).
   c. Transfer the tube to 37°C for 20 min (after 10 min, gently shake the tube).
   d. Aspirate the papain solution from the tube and wash gently once with dissection medium and once more with 10 mL of medium for glial culture.
   e. Add 1.5 mL of medium for glial culture to each cortex, mechanically dissociate twice with a glass Pasteur pipette and then tap vigorously the tube 10–20 times.
   f. Wait 1–2 min for the debris to deposit at the bottom of the tube, then filter the supernatant with a 100 μm cell strainer: filter max 5 brains *per* cell strainer.
   g. Repeat the passages e-f if the pellet is still abundant.
   h. Add medium for glial culture up to 25 mL *per* 50 mL Falcon conical tube.
   i. Plate the cells on T75 flasks using 12.5 mL of medium *per* flask (considering 2–2.5 corteces per flask).
3. Microglia isolation and plating.
   a. Leave the flasks in the CO_2 incubator at 37°C for at least 10 days and up to 14 days.
      i. By phase microscopy, microglia should appear as small, bright cells growing on top of a layer of astrocytes.
      ii. If needed, a change of medium can be performed at day 4 or 7. The medium can be changed entirely or by adding fresh medium to half volume of the conditioned medium.
      iii. The number of microglia cells should increase exponentially in the last few days in culture.
   b. At confluence (max 14 DIV), isolate microglia cells from the underlying astrocyte layer:
      i. Shake the flasks at 37°C (115–120 rpm) for 1 h and 30 min.

⚠ CRITICAL: excessive shaking can activate microglia cells. Upon shaking, most microglia cells will be in suspension, although some will remain attached to the astrocyte layer.

   c. Keep the flask horizontal and vigorously tap it twice to help cells detach.
      i. Collect the supernatant and filter it through a 70 μm cell strainer.
      ii. Spin the cells down at 1000–1200 rpm for 7 min.
      iii. Remove and save part of the medium as astrocyte conditioned medium (ACM).
   d. Disperse the pellet and estimate viable cell number using a Thoma cell counting chamber.
   e. Resuspend the desired number of cells in medium composed of half ACM and half fresh DMEM supplemented with 2.5% FBS.

⚠ CRITICAL: a higher percentage of serum may activate microglia.

   f. Plate the cells in the desired dish or plate, e.g., Ibidi μ-Slide 8 Well, pre-coated as described in the before you begin section.

i. To minimize activation due to *in vitro* culturing conditions, plate microglia at low cell density (e.g., $7 \times 10^3/cm^2$). When plating microglia cells for infection, plate $1 \times 10^4/cm^2$ in 250 μL in ibidi μ-Slide 8 Well.

4. Microglia polarization.
   a. The day after plating, cells are induced to polarize in the presence of LPS and IFNγ or IL-4 for 48 h:
   b. Add LPS at 100 ng/mL and IFNγ at 20 ng/mL to obtain activated microglia.
   c. Add IL-4 at 20 ng/mL to obtain alternatively activated microglia.

⚠ CRITICAL: Microglia polarization can be achieved by adding the cytokines directly into the medium or by removing only half of the medium and adding an equal volume of fresh medium already containing the cytokines at 2× of the final concentration.

5. Identification of reactive microglia.
   a. Morphology and gene expression should be characterized to determine the reactive state of microglia cells in culture. Refer to Rosito et al., 2023[1] for morphological characterization of activated and alternatively activated microglia.

Preparation two: Microtubule labeling and live-imaging

Timing: 3 h

6. Reconstitute SiR-tubulin and Verapamil by following the manufacturer instructions (https://www.cytoskeleton.com/pdf-storage/datasheets/cy-sc002.pdf).
7. Replace half of the medium with astrocyte conditioned medium and add SiR-tubulin to a final concentration of 100 nM and Verapamil to a final concentration of 10 μM.
8. Incubate at 37°C for 30 min.
9. If the signal is too dim, wash the cells once with conditioned medium prior to visualization to improve signal to noise ratio.
10. Acquire time lapse movies of microglia SiR-tubulin labeled microtubules, using standard Cy5 settings, maintaining the temperature at 37°C during recordings. We found the acquisition to be optimal using an Olympus IX73 microscope, LDI laser source and CoolSNAP Myo camera, 4.54 μm pixels (Photometrics) with a built-in incubator with acquisition rate of 1 frame/4 s for 4 min with a UPLSXAPO 100×/1.45 oil objective. Only one or two cells should be imaged per field.

Note: we determined that wide-field microscopy was suitable for detecting optimal signal to noise ratio by focusing on the thin edge of the cell: it is easier to identify microtubules in a distal region where the cell displays a thin edge, compared to the region proximal to the nucleus where microtubules are more densely packed (see Figure 1 as an example). Additionally, an acquisition rate of 1 frame per 4 s for a duration of 4 min was found to be optimal for minimizing photobleaching. Avoid washing steps before imaging.

Preparation three: Lentivirus preparation and microglia lentiviral infection

Timing: 3 days

11. Transfect HEK 293T cells at 60%–80% confluency with the lentiviral expression plasmid and lentiviral packaging plasmids (pLP1, pLP2, and pLP-VSV-G):
    a. For each 150 mm dish, mix 20 μg of lentiviral expression plasmid encoding EB3-EGFP pLVX-EB3-EGFP, 12 μg of pLP1, 4 μg of pLP2 and 6 μg of pLP-VSVG with sterile ddH_20 in a 5 mL polystyrene round bottom tube up to 750 μL of total volume.
    b. Add 250 μL of sterile 2 M solution of $CaCl_2$.

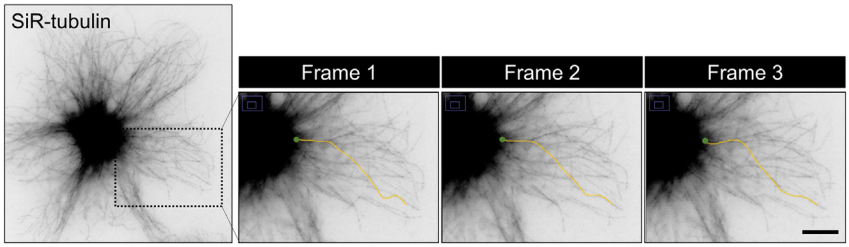

Figure 1. Microtubule tracing

Example of microtubule tracing between successive frames using the «Freehand line » tool in ImageJ from a microglia cell activated with LPS-IFNγ. Scale bar is 10 μm. The reference point is highlighted with a green dot. Note that the distal, thin edge of the cell presents more distinguishable microtubules compared to the center of the cell.

c. While gently vortexing, add dropwise 1 mL of transfection buffer 2× pre-warmed at 37°C (see composition in materials and equipment section).

Note: the reaction between Na_2PO_4 contained in the transfection buffer, $CaCl_2$ and DNA will form calcium phosphate/DNA precipitate visible at the naked eye as a faint blue tinge.

d. Quickly add the mixture to the dish of HEK 293T cells dropwise and gently agitate the dish.
e. Replace the medium with 14 mL of fresh medium at 6 h after transfection.

12. Harvest and freeze the virus:
    a. Collect the medium 12, 24 and 36 h after transfection using the same Falcon 50 mL tube, filling it up to 40 mL of medium. Harvested virus can be stored at 4°C. Filter the virus with a 0.45 μm filter system to remove debris.
    b. Add 10 mL of lentiviral precipitation solution, mixing well by inverting the tube.
    c. Leave at 4°C for at least 6 h.
    d. Centrifuge the 50 mL tube twice at 1500 × *g* for 30 min at 4°C to collect the viral pellet.
    e. Resuspend the pellet in 400 μL of medium and store at −80°C.

Note: virus titer is stable at −80°C for at least one year. Avoid multiple freeze-thaw cycles.

13. Infect microglia cells.
    a. After plating microglia cells at 1 × $10^4/cm^2$ in 250 μL as described in *preparation one*, wait at least 24 h before proceeding with the infection to let cells attach.
    b. Add 20 μL of virus for each well and wait 24–36 h before imaging.

Note: This protocol produced a viral titer of 2 * 10^8 IU/mL. In our hands, the added volume of virus (20 μL) produced the highest infection efficiency and the best signal to noise ratio for EB3-EGFP comets during live imaging. The expected outcome is described in Figure 5.

14. Acquire videos using an epifluorescence microscope. We used a Nikon Eclipse Ti equipped with an Orca II ER charge-coupled device (CCD) camera (Hamamatsu, Hamamatsu, Japan) and a temperature-controlled (37°C) CO2 incubator using a 60×/1.40NA objective at 1 frame every 2 s for at least 3 min.

Preparation four: Immunostaining of fixed microglia cells

Timing: 2 days

15. For optimal microtubule staining, fix the cells on Ibidi μ-Slide 8 Well plates with ice-cold (stored at −20°C) methanol for 4 min at −20°C and rehydrate them with PBS at R.T. for at least 30 min.
16. Block the cells with 3% BSA in PBS for 45–60 min.

17. Incubate with primary antibodies plus 1.5% BSA in PBS overnight at the following concentrations: rat tyrosinated tubulin YL 1/2, Merck-Millipore, 1:1000; mouse EB1, BD Biosciences, 1:100.
18. Wash with PBS three to four times.
19. Incubate with minimally cross-reactive fluorophore-conjugated secondary antibodies in PBS (Alexa Fluor 488 goat anti-rat, 594 goat anti-mouse) and Hoechst (Sigma-Aldrich) for nuclei visualization for 1 h at R.T.
20. Wash three to four times with PBS.
21. Mount using a few drops of Ibidi Mounting Medium.
22. Let the mounting medium sit for at least 6 h to overnight at 4°C.
23. Acquire images at a confocal microscope: we found the acquisition to be optimal using a Nikon Eclipse Ti confocal microscope equipped with X-Light V2 spinning disk (CrestOptics, Rome, Italy) and an LDI laser source (89 North, Williston, VT, USA) and Prime BSI Scientific CMOS (sCMOS) camera, 6.5 μm pixels (Photometrics) with a 100×/1.45 Plan E oil objective, with a z-step size of 0.2 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
EB1 (mouse monoclonal) 1:100	BD Biosciences	Cat#610535; RRID AB_397892
Tyr tubulin (rat monoclonal) 1:1000	Millipore	Cat#MAB1864; RRID AB_2128189
Alexa Fluor 488 goat anti-rat 1:500	Invitrogen	Cat#A11006; RRID AB_141373
Alexa Fluor 594 goat anti-mouse 1:500	Invitrogen	Cat#A11032; RRID AB_2534091
Chemicals, peptides, and recombinant proteins		
Murine recombinant IL-4	PeproTech	Cat# 214-14
Murine recombinant IFNγ	PeproTech	Cat# 315-05
LPS from *E. coli*	Sigma-Aldrich	Cat# L4391
Hoechst	Sigma-Aldrich	Cat# 23491-45-4
Poly-L-lysine hydrobromide	Sigma-Aldrich	Cat# P6282
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich	Cat# D6546
Fetal bovine serum (FBS)	Gibco	Cat# 11573397
Lentiviral precipitation solution	ALSTEM	Cat# VC100
Critical commercial assays		
SiR-Tubulin Kit	Spirochrome	Cat# CY-SC002
Experimental models: Cell lines		
HEK 293T	ATCC	CRL-3216
Experimental models: Organisms/strains		
Mouse: C57BL/6 pups post-natal day 0–2	Charles River Laboratories	027C57BL/6
Recombinant DNA		
EB3-EGFP	Pero et al.[8]	N/A
pLP1, pLP2, and pLP-VSVG	Thermo Fisher	N/A
Software and algorithms		
GraphPad Prism v9.0	GraphPad	https://www.graphpad.com
FIJI/ImageJ 1.53c	Schindelin et al.[10]	http://www.imagej.nih.gov/ij
Other		
μ-Slide 8 Well	ibidi	Cat# 80826
ibidi Mounting Medium	ibidi	Cat# 50001

MATERIALS AND EQUIPMENT

For primary microglia cell culture preparation.

- Poly-L-lysine solution: 5 mg to dissolve in 50 mL of ddH_2O.

Prepare fresh or store at −20°C up to 1 month.

- Dissection medium: HBSS with 10 mM HEPES.

Store at 4°C for a maximum of 3–4 weeks.

- Medium for glial culture: DMEM supplemented with 10% FBS.

Store at 4°C for a maximum of 3–4 weeks.

Papain solution		
Reagent	Final concentration	Amount
Papain 16 units/mg	N/A	30 µL
EDTA (50 mM)	0.5 mM	15 µL
$CaCl_2$ (150 mM)	1.5 mM	15 µL
Cysteine 20 mg/mL	0.2 mg/mL	15 µL
DNase 0.1 mg/mL	0.1 µg/mL	1.5 µL
Dissection medium	N/A	1.42 mL
Total	**N/A**	**1.5 mL each cortex**
Prepare freshly before dissection in sterile conditions, keep at R.T. until use.		

For lentivirus preparation.

- HEK 293T media: DMEM with 10 mM HEPES and 10% BCS

Transfection buffer 2×		
Reagent	Final concentration	Amount
HEPES	55 mM	6.553 g
NaCl	270 mM	7.889 g
$Na_2PO_4 \cdot 2H_2O$	1.5 mM	0.133 g
Total	**N/A**	**500 mL**
Sterilize and adjust pH = 7.05. Store in aliquots at −20°C. Multiple freeze-thaw cycles do not affect transfection efficiency.		

⚠ CRITICAL: Follow institutional approved guidelines and use certified personal and collective protective equipment for virus handling.

STEP-BY-STEP METHOD DETAILS

Live cell analysis: Sir-tubulin labeled microtubule length tracing

Timing: 2 h (*per* time-lapse movie)

This step enables the analysis of microtubule dynamics by tracking changes in microtubule length between successive frames of a time-lapse movie. The analysis accounts for potential bending or movement of the microtubule and can be performed using tools available in ImageJ software.

Note: This analysis has been adapted from Dhamodharan and Wadsworth 1995,[12] Dhamodharan et al., 1995,[14] Rusan et al. 2001.[16]

For image preparation, use ImageJ for level adjustments of frame brightness and contrast, and background subtraction.

1. Microtubule tracing:
   a. Use ImageJ to open the movie obtained by recording SiR-tubulin labeled microglia.
   b. Invert LUT using ImageJ dedicated command.
   c. Select a "substack" to extract from a stack a selected image in which microtubules are clearly visible.
      i. From "Image" menu, select "Stacks", then "Tools" and "Make substack".
   d. Select one microtubule that is visible for the duration of the entire substack.
   e. Choose a reference point that is stable for the duration of the entire substack and make a note of its XY coordinates.
   f. Starting from the reference point, trace a line using the "Freehand line" tool, outlining the microtubule entire length.
   g. If needed, draw the microtubule for future reference selecting "Draw" from the "Edit" menu.
   h. Measure the length of the selection with the "Measure" command in the "Analyze" menu.
   i. Change frame and repeat, making sure to trace the line from the same reference point, until the end of the "substack".
2. Report the measurements and measure microtubule dynamics parameters:
   a. Copy the values into an Excel file.
   b. Starting from the second frame, measure the difference in length with the previous frame.
   c. For each difference value, note if it represents a "growth" (positive value), "shrinkage" (negative value) or "pausing" (value $\leq$ 0.5 μm) event.
   d. For each "growth" or "shrinkage" event, divide the absolute value by the time interval between frames to obtain a "growth" or "shrinkage" rate in μm/s, and average all values to obtain an "average growth" or "shrinkage rate" in μm/s.
   e. Count the number of "growth", "shrinkage" or "pausing" events and divide each one by the number of total events ("growth" + "shrinkage" + "pausing") to obtain a percentage of "growth", "shrinkage" and "pausing".
   f. Calculate the total time of analysis (in seconds) by multiplying the number of frames by the time interval between frames.
   g. Count the number of transitions between "growth" and "shrinkage" (catastrophe) and vice versa (rescue).
   h. Calculate "catastrophe" or "rescue frequency" by dividing the number of "catastrophe" or "rescue" events by the product between the percentage of "growth" or "shrinkage" and the total time of analysis.
   i. Calculate microtubule "dynamicity" by dividing the sum of all the "growth" or "shrinkage" event absolute numbers by the total time of analysis.

⚠ CRITICAL: Draw the line as carefully as possible, comparing successive frames through the entire video to follow the behavior of the entire microtubule. This will help discriminate the microtubule when the frame is blurred or unclear. See Figure 1 as an example.

Note: As an indication, at least 30 substacks per condition from three independent culture experiments should be analyzed for a treatment comparison.

Live cell analysis: EB3-EGFP comet kymograph analysis

Timing: 2 h (*per* cell)

This widely used procedure allows for the analysis of microtubule plus end dynamics and orientation in ramified segments of homeostatic microglia.

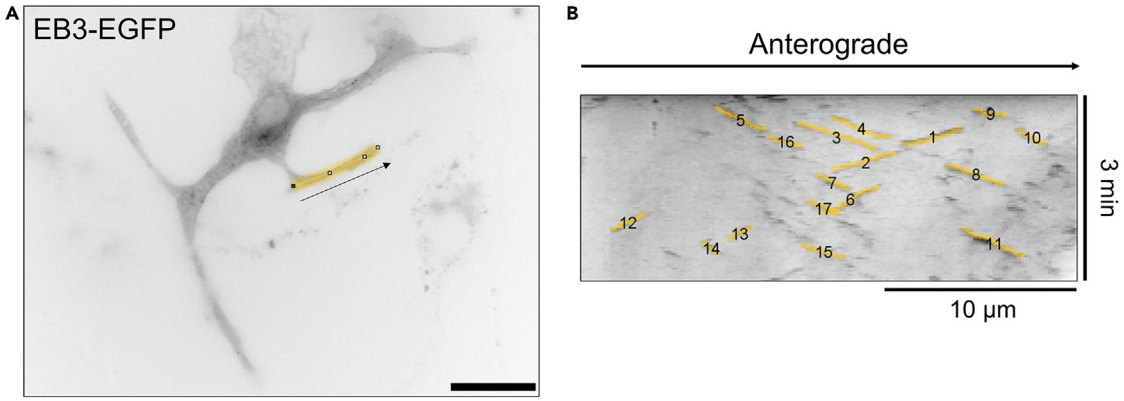

Figure 2. Example of *Projected kymograph* and outline of *Complete comets*

(A) Frame from a movie obtained from EB3-EGFP expressing homeostatic microglia (Scale bar: 20 µm). The yellow line is obtained with the «Segmented line » tool, with known width and indicates the region from which the kymograph is obtained in B. The direction of the drawn line is highlighted by the arrow and will determine the direction of the comets in the kymograph in B.

(B) *Projected kymograph* with *Complete comets* highlighted in yellow and numbered with the ImageJ ROI manager. Comets number 1, 2, 6 and 13 are defined as Retrograde.

Note: the signal of microtubule plus end binding (EB) proteins (later defined as "comet") typically decays exponentially after the maximum at the microtubule end that is toward the microtubule lattice.[17,18]

3. Obtain a kymograph from an EB3-EGFP microglia movie:
   a. Use ImageJ to open the movie obtained by recording EB3-EGFP comets in expressing microglia.
   b. Invert LUT using ImageJ dedicated command.
   c. Select a "substack" based on the planes where the signal is bright and the EB3 comets are clearly trackable.
   d. With the "Segmented line" tool, select the section that will be analyzed by the Kymograph plugin.

   Note: Adjust the line width to cover the region that will be analyzed and make a note of the value.

   e. From the ImageJ Plugin menu, select "Kymograph" and "Kymograph builder".

   Note: The resulting kymograph displays the line length as X, and the time as Y (as displayed in Figure 2). Make sure the values are based on the correct pixel-micron conversion by checking the original file properties from "Image" menu.

4. Calculate the percentage of anterograde and retrograde comets.

   Note: The direction of the drawn line will determine the direction of the comets (as explained in Figure 2): by drawing the line from the proximal part of the process towards the distal part with respect to the nucleus, the resulting kymograph will display anterograde comets as directed from left to right, while retrograde comets will be directed from right to left (Figure 2B).

5. Comet analysis (optional):
   a. Report on an Excel file the *Frame interval*, the *Number of analyzed frames* and the *Duration of the analyzed substack*, obtained by multiplying the number of analyzed frames for the frame interval.

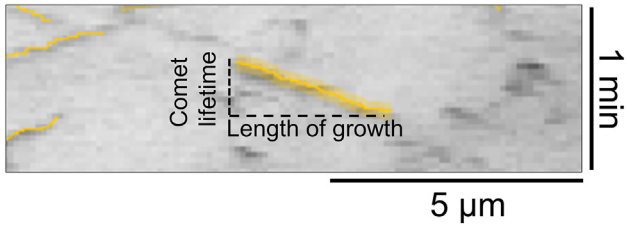

Figure 3. Example of Comet lifetime and Length of growth measurement X and Y projections of the comet (highlighted in yellow) represent Length of growth and Comet lifetime, respectively (dashed lines, black)

b. Report the analyzed cell *Process length* and *Process width* in µm, defined by the length and the width of the drawn line, respectively.
c. On the *Projected kymograph*, outline with the "Freehand line" tool the complete comets to be analyzed and add them to the ROI Manager, from the "Tools" section in the "Analyze" menu. See Figure 2 for an example of *Projected kymograph* and *Complete comets*.

Note: Comets are defined as "complete" if not intersecting with the borders of the *Projected kymograph* and the start and the end of it are clearly identifiable.

6. Microtubule dynamics analysis (optional):
   a. For each comet outlined in the *Projected kymograph*, measure *Length of growth* (covered distance on the X axis) and *Comet lifetime* (projection on the Y axis of the comet trajectory) as in Figure 3.
   b. Convert the value of the *Comet lifetime* from microns to seconds.

Note: The width of the drawn line in pixels (*Process width* in pixels) will define the number of pixels corresponding to the Y axis of the kymograph; by multiplying this number for the pixel dimensions in microns (determined by the camera and the objective used during acquisition) the *Value of the Y axis of the kymograph* (microns) will be obtained. The conversion of the *Comet lifetime* value from micron to seconds will be therefore obtained as [(*Comet lifetime* in microns * *Duration of analyzed substack* in seconds) / *Value of the Y axis of the kymograph* in microns].

   c. Calculate comet *Growth rate* as *Length of growth* / *Comet lifetime* and the other desired parameters accordingly.

Note: the number of kymographs that provide a statistically valid sample size will be dependent on the length of the lines that can be drawn to cover the extension of a process (or most of the cell area in the case of an amoeboid cell). Approximately, one should collect a number of kymographs to cover at least 10 processes per experiment (e.g., at least 2 kymographs per microglia, 5 microglia per experiment) and three independent experiments.

Fixed cell analysis: EB1 fluorescence gradient analysis

Timing: 2 h

The comet-shaped accumulation of EBs at microtubule ends, with the intensity of comet tail decaying exponentially from the maximum at the microtubule end, enables the measurement of fluorescence intensity gradients of EB-positive comets relative to the location of the nucleus. Although live imaging of EBs remains the gold standard technique, this approach is straightforward and reproducible in our hands. This method enables the definition of anterograde or retrograde comets by using the fluorescence intensity gradient of endogenous EB protein signal from immunolabeled fixed cells. This method can also be applied to single frames of EB3 movies.

7. Open images of EB1 and Tyr-tubulin from immunostained microglia cells in ImageJ.
8. Using the signal of Tyr-tubulin as a reference, identify EB1 comets at the plus end of tyrosinated microtubules.
9. Measure fluorescence intensity signal of EB1 at the tip of the microtubule defined by Tyr-tubulin:
   a. Open the EB1 channel.
   b. Invert LUT.
   c. With the "Freehand line" tool, draw a line starting from the end of the comet proximal to the nucleus.

   Note: depending on the microtubule distribution and on the cell shape, the fluorescence gradient of different comets may occur at varying degrees relative to the direction or trajectory: draw the lines accordingly. Select as many clearly detectable (e.g., bright signal at the maximum of the tip of the comet, with comet tail intensity decaying exponentially) comets as possible. At least 100 comets will be needed from at least three independent experiments.

   d. Increase the line width to cover the width of the comet.
   e. With the "Plot profile" command from the "Analyze" menu, obtain a line scan of the selection for each comet.
   f. If the peak of the line scan is in the second half of the plot, define the comet as Anterograde. Otherwise, define as Retrograde, as in Figure 4.

EXPECTED OUTCOMES

By following the Sir-Tubulin analysis protocol, it is possible to measure changes in microtubule length in microglia cells, which are known to be resistant to transfection and lentiviral infection. This allows for the analysis of microtubule dynamics while accounting for potential bending and/or movement of the microtubule.

Lentiviral infection of microglia with EB3-EGFP may result in low efficiency due to the tendency of microglia cells to activate and react to infections. Nevertheless, this protocol enables the identification of growing microtubule plus ends in infected microglia, which appear as a bright, elongated comets, compared to the lower intensity of intracellular fluorescent puncta (see Figure 5). It should be noted that LPS-IFNγ activated microglia are particularly difficult to infect. Using this protocol, it is possible to identify retrograde EB3 comets in microglia ramifications and analyze their dynamic behavior in living cells. In fixed cells, the protocol is applicable to minimally manipulated cells and allows for the identification and quantification of both anterograde and retrograde EB1 comets.

LIMITATIONS

By following this protocol, it is possible to analyze different parameters that describe the dynamics and orientation of microtubules and growing microtubule plus ends in microglia cells.

SiR-Tubulin labeling of microglia microtubules may result in dim signals, that we could image only for a short period of time (4 min) before photobleaching occurs. Also, during imaging, microtubules can drift out of focus, making the analysis challenging. Prolonged exposure to Taxol derivatives, such as SiR-tubulin, may alter the dynamicity of the microtubules: we found 4–5 min of imaging with 100 nM of SiR tubulin as a reasonable time frame to avoid this problem. It should also be noted that SiR-tubulin labels all microtubules, including depolymerizing and pausing, whereas comet analysis only labels growing microtubule plus ends due to the kinetics of EB protein binding. This can result in different dynamicity values arising from the two analyses that are not directly comparable.

In our hands, EB3-EGFP lentiviral infection of microglia cells resulted in a low percentage of EB3-expressing cells (around 1%) even when varying the amount of virus and incubation time after the

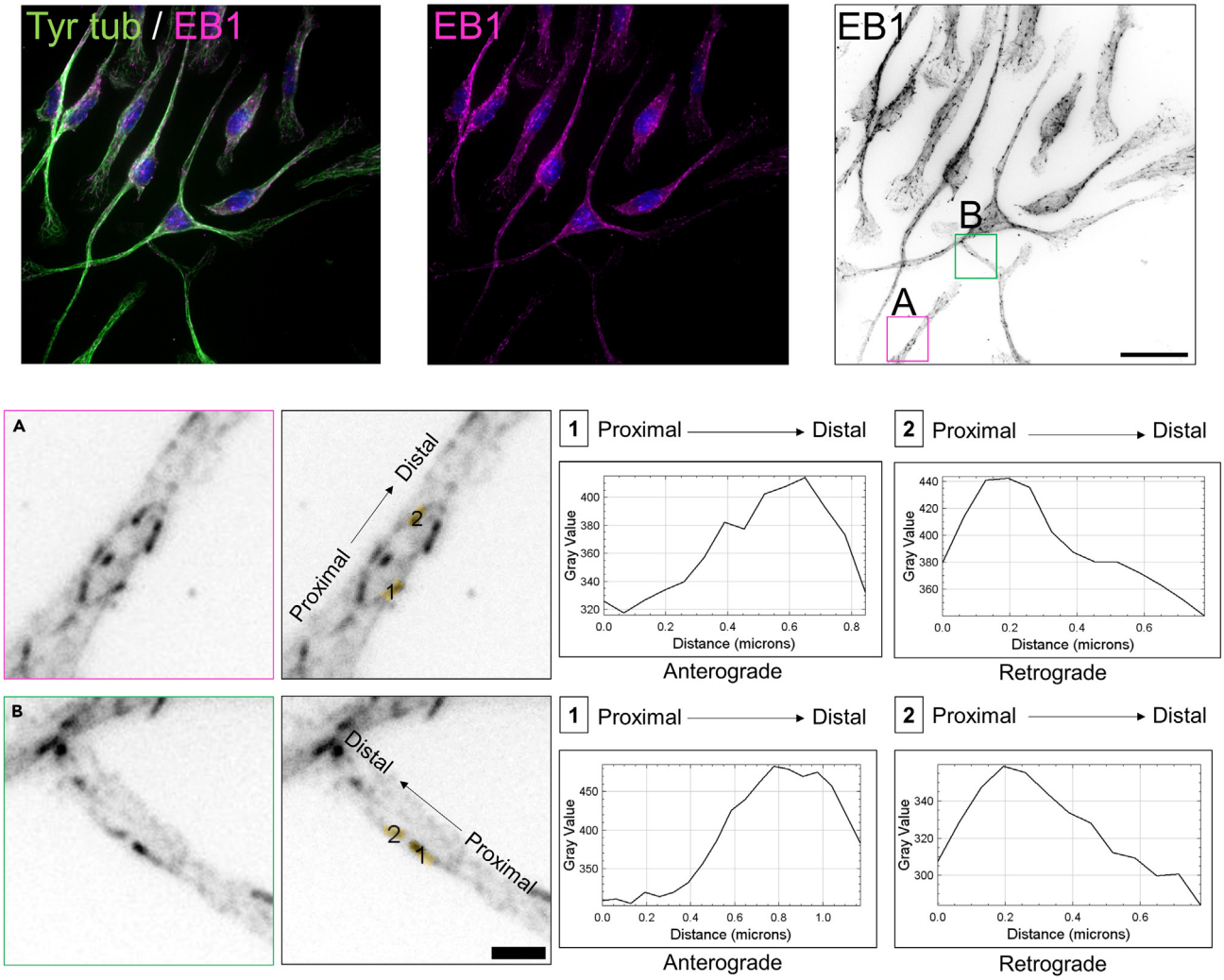

Figure 4. Analysis of EB1 fluorescence intensity gradients

Example of analysis of EB1 fluorescence intensity gradients from homeostatic microglia. *Top:* representative image of microglia immunolabeled for tyrosinated α-tubulin (Tyr tub, green) and microtubule end binding protein EB1 (magenta), Hoechst for nuclei visualization (blue), and EB1 inverted LUT signal (*left*); Scale bar: 20 μm.

Bottom: zoom of regions A (magenta) and B (green) highlighted in EB1 inverted contrast image (scale bar: 2 μm) and relative analysis: a line is drawn following the direction of the arrow, from the proximal to the distal part of the cell with respect to the nucleus. If the peak of the line scan is in the second half of the graph, the comet is defined as Anterograde (A1, B1), otherwise as Retrograde (A2, B2).

infection: therefore, the cells that are not expressing EB3-EGFP might represent a specific subtype of microglial state (e.g., activated). Indeed, we did not succeed in infecting LPS-IFNγ activated cells and could only analyze comets from homeostatic and alternatively activated cells. As mentioned before, photobleaching may occur after 3 min of live cell imaging also for these cells.

TROUBLESHOOTING

Problem 1

Low yield of microglia culture (Related to preparation one: primary microglia cell culture).

Potential solutions

- The optimal age of the pups is postnatal day 0 to postnatal day 1. The use of younger animals may result in a better yield.

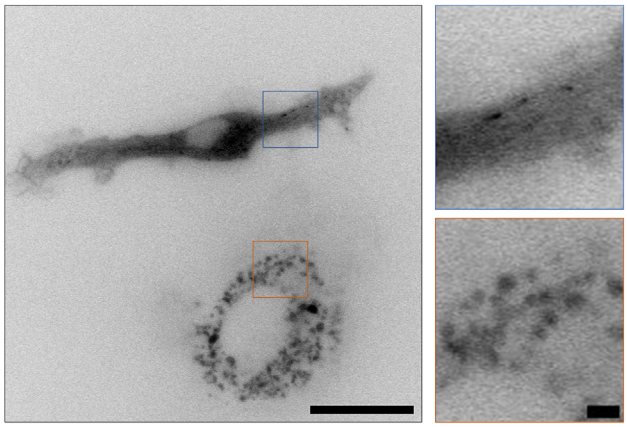

Figure 5. Example of EB3 comets recognition in EB3-EGFP expressing microglia

Example of EB3-EGFP expressing microglia cell from an untreated (homeostatic) culture. Scale bar: 20 μm. It should be easy to identify EB3 comets (highlighted in the blue zoom) from fluorescent puncta (orange zoom image); scale bar: 2 μm. Note that the puncta highlighted in the orange zoom come from an amoeboid (likely activated) cell. Activated cells display an increased number of fluorescent puncta that may hinder the identification of EB3 comets. One should consider analyzing these cells too as a distinct pool of "activated" cells.

- Use fresh solutions and keep the tissues on ice for the duration of the dissection. Dissect the brain as quickly as possible, and rapidly plate the cells in the final medium at 37°C, without leaving them on ice, in the dissection medium, or at R.T. for too long. Adjust the number of cortices per flask according to the yield obtained by the operator. A mixed glia culture (up to DIV 14) is illustrated in Figure 6.

Problem 2

High levels of cellular debris (Related to preparation one: primary microglia cell culture).

Potential solution

Reduce the trituration obtained by mechanical dissociation. Reduce the volume of enzyme added to the papain solution. Freshly prepare papain solution and medium for each dissociation. Do not filter more than 5 cortices with one cell strainer.

Problem 3

Low attachment of cells (Related to preparation one: primary microglia cell culture).

Potential solution

Carefully coat all the flasks and wells. Use freshly prepared poly-L-lysine solution and freshly coated supports.

Problem 4

Poor microtubule labeling (Related to preparation two: microtubule labeling and live imaging).

Potential solutions

- Increase the time of incubation with the probe or its wash off to increase the signal-to-noise ratio.
- Keep the labeling solution and the labeled cells in the dark. Label the cells right before imaging. Minimize the time during which cells are exposed to the light of the microscope and adjust light source power to avoid photobleaching.
- Refer to the manufacturer instructions for probe reconstitution, storage, handling and labeling for optimal results. Add Verapamil accordingly to manufacturer instructions to inhibit efflux pumps and improve labeling.

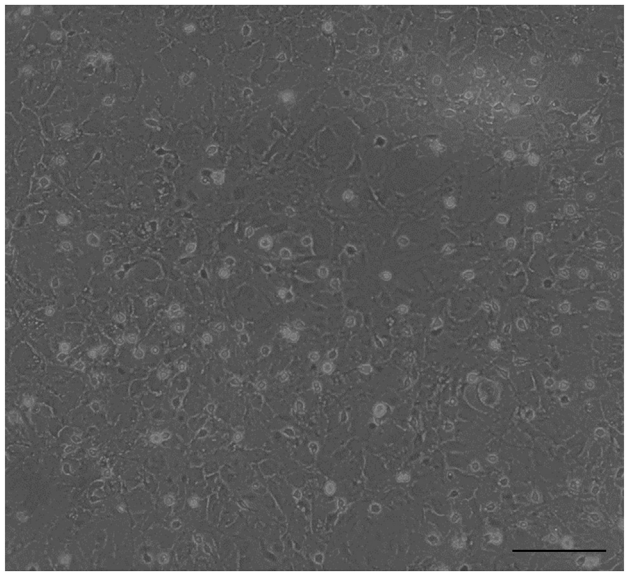

Figure 6. Example of mixed glia culture

Example of DIV 12 mixed glia culture. Note the astrocyte layer and the bright microglia cells laying on top of it. Scale bar: 100 μm.

Problem 5

Poor efficiency of lentiviral infection (Related to preparation three: lentivirus preparation and microglia lentiviral infection).

Potential solution

Since the microglia culture, especially when activated, is refractory to lentiviral infection and the viral preparations may differ in titer, we suggest increasing the volume of virus used and/or by removing some medium before the infection. Increasing the density of the microglial culture would also improve infection. Note however that higher microglia density may also lead to a higher degree of *in vitro* microglia activation.

Problem 6

Poor comet and/or microtubule labeling (Related to preparation four: immunostaining of fixed microglia cells).

Potential solutions

- Fix with a high volume of ice-cold methanol to preserve microtubule integrity during fixation and wash well for proper re-dehydration before immunolabeling.
- Freshly prepare BSA and antibody solutions and wash well when needed, being careful not to let cells detach.
- Let the mounting medium sit at least overnight, then image the sample as soon as possible to avoid photobleaching.
- Store the sample in the dark at 4°C, wrapped in parafilm to avoid evaporation.

Problem 7

Problems in visualizing microtubule movement (Related to live cell analysis: Sir-tubulin labeled microtubule length tracing).

Potential solution

Because it can be hard to visualize microtubule movement between frames, always keep the entire stack to be able to go back and forth and identify the movement. Add the traced microtubule section to the ROI manager to keep track of the movement between frames.

Problem 8

Problems in identifying the fluorescence intensity gradient of EB comet (Related to fixed cell analysis: EB1 fluorescence gradient analysis).

Potential solution

Measure the same comet multiple times, slightly changing the selection of the line scan to make sure to identify the peak of fluorescence intensity. Make sure to cover the entire length and width of the comet when measuring fluorescence intensity to average the signal correctly.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Silvia Di Angelantonio (silvia.diangelantonio@uniroma1.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

F.B., S.D.A., and M.R. designed the study. C.S., M.R., and A.C. performed experiments and generated primary data. S.D.P. provided helpful insights and contributed key techniques. C.S. performed data analysis. S.D.P., F.B., and S.D.A. supervised data analysis and experiments.

DECLARATION OF INTERESTS

S.D.A. is a member of the scientific advisory board of D-Tails s.r.l. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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