Protocol

Live imaging of microtubule dynamics at excitatory presynaptic boutons in primary hippocampal neurons and acute hippocampal slices

Analyses of microtubule (MT) plus end dynamics at glutamatergic en passant boutons can be carried out in cultured primary neurons isolated from mouse or rat embryos or ex vivo in acute slices isolated from mice that had been electroporated in utero. Here, we describe a protocol for setting up and analyzing live image recordings of primary neurons and acute hippocampal slices expressing tagged versions of the MT plus end binding protein EB3 and the presynaptic vesicle markers vGlut1 or VAMP2.
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Live imaging of microtubule dynamics at excitatory presynaptic boutons in primary hippocampal neurons and acute hippocampal slices

Xiaoyi Qu,1,2,3 Atul Kumar,1,3 and Francesca Bartolini1,4,*

1Department of Pathology & Cell Biology, Columbia University Medical Center, New York, NY 10032, USA
2Present address: Genentech, Inc., South San Francisco, CA 94080, USA
3Technical contact
4Lead contact
*Correspondence: fb2131@columbia.edu
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SUMMARY
Analyses of microtubule (MT) plus end dynamics at glutamatergic en passant boutons can be carried out in cultured primary neurons isolated from mouse or rat embryos or ex vivo in acute slices isolated from mice that had been electrooporated in utero. Here, we describe a protocol for setting up and analyzing live image recordings of primary neurons and acute hippocampal slices expressing tagged versions of the MT plus end binding protein EB3 and the presynaptic vesicle markers vGlut1 or VAMP2.

For complete information on the use and execution of this protocol, please refer to Qu et al. (2019).

BEFORE YOU BEGIN
Experimental considerations
There are two potential starting points: primary neurons or acute slices.

Primary neurons have the following advantages: (1) less laborious; (2) the success rate does not depend on animal survival; (3) faster image acquisition (fewer z-stacks required) to capture all MT nucleation/rescue events; (4) easy for tracking both the proximal and distal portion of the axon; (5) lower chance of interfering with baseline MT dynamics by shorter EB3-EGFP expression. However, the disadvantages are: (1) the circuit is not intact; (2) embryonic neurons; (3) healthy cultures need to be maintained for several weeks in vitro; (4) no correlation with behavioral readouts.

Note: To follow the protocol using primary neurons, follow steps 1–9 in Before you begin and then proceed to 1–7 in Step-by-step method details.

Acute slices have several advantages: (1) spatial compartmentalization is maintained; (2) genetically engineered animals can be used; (3) acute slices are isolated from P21-P28 developed brains in contrast to cultured neurons that are derived from embryos; (4) no high-resolution live-imaging approach is available to perform optical studies in the hippocampus of live animals so acute slices offer a valuable alternative; (5) can be coupled with electrophysiological recordings of specific circuits. The disadvantages are: (1) more laborious and relies on the overexpression of wild-type or mutant genes that are hard to transfect or infect using viral particles ex vivo; (2) the success rate depends on mouse survival from electroporation in utero; (3) not easily adapted for high throughput studies or drug screenings; (4) no correlation with behavioral readouts.
**Primary rat hippocampal neuronal cultures**

**Day 1**

- **Timing:** 10 min

1. Coat MatTek live-imaging dishes (35 mm Petri dish, 14 mm microwell) with 0.1 mg/mL poly-D-lysine (PDL) in 1/3 sterile borate buffer for 24–48 h at 37°C. The microwell holds ~300 μL volume. PDL only needs to cover the microwell.

**Day 2**

- **Timing:** 2–3 h

2. Wash microwells 3 times with ddH₂O.

   **Pause point:** If not seeding cells immediately, cover the microwells with culturing medium. Do not let them dry.

   Culture primary hippocampal neurons from E18 rats. Seed 50,000–70,000 cells into microwells of coated MatTek dishes. Spread the cells evenly in the center. After 2 h, add 2 mL of culturing medium.

**Days 3–18**

- **Timing:** 30 min/medium change

3. To maintain the cultures, change medium every 3–4 days. If starting volume is ~2 mL, take 300–400 μL out and add 500–600 μL of conditioned medium.

   **CRITICAL:** Using conditioned medium collected from dense cultures (100,000 neurons/cm²) is critical during the first 2 weeks of culturing. Conditioned medium is composed of growth medium from dense cultures at the same DIV and fresh growth medium at 1:1 volume ratio.

   **CRITICAL:** When adding or removing volumes from the dish, pipette from the side. Do not pipette directly into the microwell.

4. Healthy neurons will be evenly spread in the imaging chamber with multiple extended and branched dendrites and high spine density (0.5–0.8 spine/μm). This is critical for transfection efficiency and expected EB3 puncta motility (Figure 1).

   **CRITICAL:** If the neuronal culture does not appear homogenously healthy prior to transfection, trash the cells and do not proceed to the next step. Unhealthy neurons will have short or very few dendritic branches, aggregated neurites, or lift off from the bottom of the well.

**Transfection of primary rat hippocampal neurons**

Using this protocol, the transfection efficiency of cultured neurons at 18 DIV is typically no higher than 1% regardless of density of plating, offering the advantage to readily identify single axons even in high density cultures.
Day 19

© Timing: 2.5–3 h

5. Hippocampal neurons are transfected at 18 DIV with Lipofectamine 2000 reagent. For one dish, mix 0.5–2.5 μg of plasmid DNA with 300 μL of incomplete neurobasal medium (no B27, no glutamax, no antibiotics → critical for transfection efficiency). For co-transfection, mix the 2 plasmid DNAs together into incomplete neurobasal medium at this step.

**Note:** plasmid DNAs were made from low endotoxin maxiprep kit for example PureLink HiPure Plasmid Filter Maxiprep Kit and were titrated for each batch to make sure cells are expressing the proper level of proteins. Usually for EB3-EGFP (gift from Franck Polleux), use 1.2–1.5 μg/dish; VAMP2-mCherry (gift from Clarissa Waites) or vGlut1-mCherry (gift from Clarissa Waites), 0.5–0.8 μg/dish. EB3 expression should be bright enough to detect comets during live imaging, while having low cytosolic background. Similarly, for VAMP2 or vGlut1 expression, boutons should appear as bright puncta without interfering with the comet signals in the other channel.

6. For one dish, add 3 μL of Lipofectamine 2000 to the diluted plasmid DNA-media mix followed by vortexing. Incubate Lipofectamine-DNA mix at 20°C–25°C for 30 min.

7. Pipette original conditioned medium out from the dishes and save in conical tubes. Add lipofectamine-plasmid DNA mix dropwise to the microwells and make sure the microwells are well covered. Put dishes back into the incubator and the conditioned medium in conical tube in a 37°C water bath.

8. Incubate for 2 h.
9. Take dishes out of the incubator and aspirate out transfecting reagents. Tilt the dish so that the liquid is aspirated out completely and the pipette tip does not touch the cells. Add back 2 mL of conditioned medium immediately. Do not let the cells dry.

**Brain in utero electroporation followed by acute hippocampal slicing**

*Note:* This protocol is adapted from Stoppini et al. (1991).

10. Monitor breeding females daily to assess for vaginal plugging and timed pregnant mouse at E15.5.

⚠️ CRITICAL: The success of the procedure can be highly strain dependent, i.e., B6 mice are not good at caring for the pups after birth. We cross SV129 with B6 and use the F1s for most of our procedures but CD1 also work well.

11. Prepare plasmid DNA (1–2 μg/μL) in 0.5% Fast green dye (Sigma-Aldrich).
12. Prepare a pulled glass pipette (6 inch; OD/ID: 1/0.58) using dual stage glass micropipette puller (PC-10; Narishige’s).
13. Weigh the mouse and give the mouse drug injections:
   a. Subcutaneous Buprenex – 0.1 mg/kg
   b. Subcutaneous Rimadyl – 5 mg/kg

*Note:* The use of drugs depends on institution animal rules.

14. Inject a mix of endotoxin-free plasmid preparation and Fast Green into one lateral hemisphere of E15.5 embryos using a Picospitzer III (Parker).
15. Electroporation (ECM 830, BTX) is performed to target hippocampal progenitors in E15.5 embryos by placing the anode (positively charged electrode) on the side of DNA injection and the cathode on the other side of the head.
16. Five pulses of 45 V for 50 ms at 500 ms intervals are used for electroporation.
17. After electroporation, it is best not to touch the mouse/change the cage following the injections until a few days after birth (usually around 5–7 days after birth).

**Acute hippocampal slices preparation**

*Note:* This protocol is adapted from Stoppini et al. (1991) and Yasumatsu et al. (2008).

18. Anesthetize 21–28 days old electroporated mice with 5% isoflurane.
19. Check for the absence of reflex (tail or paw pinching) before decapitation.
20. Remove the brain from the animal: Slice the scalp down the midline with a scalpel, and then carefully cut the skull along the midline with a fine pair of scissors, taking care to not cut the underlying brain tissue.
21. Transfer the brain in ice cold cHBSS buffer.
22. In the case of the hippocampus: place the brain ventral side down, locate the superior colliculi, make a transverse cut, and discard the caudal part.
23. Flip the brain ventral side up and make a transverse cut.
24. Spread glue on the cutting plate of vibratome and gently lift the brain with spatula and place it over glue with brain rostral side up and ventral side facing you.
25. Transfer slicing chamber in vibratome, lock it, and pour cHBSS to submerge the brain.
26. Prepare the vibratome by placing a mix of ice and water in the tray surrounding the slicing chamber. Cut a razor blade in half and place one half in the blade holder of the vibratome.

27. 200 µm thick slices (speed of the vibratome 0.08–0.1 mm/s) are prepared with a vibratome (Leica Biosystem VT 1000S) and collected in 6 well plates with complete cHBSS media.

28. Transfer acute slices into 35 mm MatTek dishes for live imaging.

△ CRITICAL: Live cell imaging of EB3 comets in acute hippocampal slices is performed immediately after preparation of slices.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE       | SOURCE                        | IDENTIFIER     |
|---------------------------|-------------------------------|----------------|
| Recombinant DNA           |                               |                |
| EB3-EGFP                  | Gift from Franck Polleux     | N/A            |
| vGlut1-mCherry            | Gift from Clarissa Waites    | N/A            |
| VAMP2-mCherry             | Gift from Clarissa Waites    | N/A            |
| Chemicals, peptides, and recombinant proteins |                     |                |
| Lipofectamine 2000        | Thermo Fisher                 | 11668019       |
| D-Glucose                 | Sigma-Aldrich                 | G8270          |
| CaCl2                     | Sigma-Aldrich                 | C5670          |
| MgSO4                     | Sigma-Aldrich                 | M2643          |
| NaHCO3                    | Sigma-Aldrich                 | 144-55-8       |
| Neurobasal media          | Thermo Fisher                 | 1103049        |
| B-27 supplement (50×)     | Thermo Fisher                 | 7504044        |
| GlutaMAX supplement       | Thermo Fisher                 | 35050061       |
| Penicillin-streptomycin   | Thermo Fisher                 | 15140163       |
| Trypsin 0.05% EDTA        | Thermo Fisher                 | 25300054       |
| 10× HBSS                  | Thermo Fisher                 | 14065056       |
| 1 M HEPES (pH7.4)         | Thermo Fisher                 | 15630080       |
| Poly-D-lysine             | Sigma-Aldrich                 | P1149          |
| D-APS                     | Sigma-Aldrich                 | A8054          |
| Bicuculline               | Sigma-Aldrich                 | 14343          |
| Ammonium chloride         | Sigma-Aldrich                 | 254134         |
| BDNF                      | R&D Systems                   | 248-8DB        |
| Fast green                | Sigma-Aldrich                 | F7252          |
| Isoflurane                | Primal Healthcare             | 66794-017-25   |
| Critical commercial assays|                               |                |
| PureLink HiPure Plasmid Maxiprep Kit | Thermo Fisher | K210007       |
| Experimental models: organisms/strains |              |                |
| DH5a                      | New England BioLabs           | C2987H         |
| Mouse: C57BL/6J           | Charles River Laboratories    | RRID:IMSR_CRL.027 |
| Rat: Sprague-Dawley       | Charles River Laboratories    | RRID:RGD_734476 |
| Software and algorithms   |                               |                |
| ImageJ (Fiji)             | NIH                           | RRID: SCR_002285 |
| MetaMorph microscopy automation and image analysis software | Molecular Devices | RRID: SCR_002368 |
| Andor iQ3                 | Oxford Instruments            | RRID:SCR_014461 |
| GraphPad Prism            | GraphPad                      | RRID: SCR_002798 |
| Nikon Elements software   | Nikon Instrument              | RRID:SCR_014329 |
| Other                     |                               |                |
| 35 mm MatTek dishes       | Mattek corporation            | P3SG-1.5-14-C  |
| Millicell membrane        | Millipore                     | HAWP02500      |

(Continued on next page)
MATERIALS AND EQUIPMENT

Neuronal culture medium

| Component           | Stock concentration | Final concentration | Volume  |
|---------------------|---------------------|---------------------|---------|
| Neurobasal medium   | 1 x                 | 1 x                 | 483.75 mL |
| B-27 supplement     | 50 x                | 1 x                 | 10 mL   |
| Pen/strep           | 100 x               | 1 x                 | 5 mL    |
| GlutaMAX            | 200 mM              | 0.5 mM              | 1.25 mL |
| Total               |                     |                     | 500 mL  |

Aliquot in 50 mL conical tubes and store at 4°C. Use within 1 week.

Complete HBSS (cHBSS)

| Component          | Stock concentration | Final concentration | Volume  |
|--------------------|---------------------|---------------------|---------|
| HBSS               | 10 x                | 1 x                 | 50 mL   |
| HEPES (pH 7.4)     | 1 M                 | 2.5 mM              | 1.25 mL |
| D-Glucose          | 1 M                 | 30 mM               | 15 mL   |
| CaCl2              | 100 mM              | 1 mM                | 5 mL    |
| MgSO4              | 100 mM              | 1 mM                | 5 mL    |
| NaHCO3             | 1 M                 | 4 mM                | 2 mL    |
| ddH2O              |                     |                     | 421.75 mL |
| Total              |                     |                     | 500 mL  |

Sterile filter with a 0.2 μm filter. Store at 4°C.

Microscope setup

Live imaging in cultured hippocampal neurons is performed on IX83 Andor Revolution XD Spinning Disk Confocal System. The microscope is equipped with a 100×/1.49 oil UApO objective, a multi-axis stage controller (ASI MS-2000), and a controlled temperature and CO2 incubator. Movies are acquired with an Andor iXon Ultra EMCCD camera and Andor iQ 3.6.2 live cell imaging software at 0.5–2 s/frame for 3 min.

Live imaging in acute hippocampal slices is performed with a Nikon A1R GaAsP multi detector unit on an inverted Nikon Ti-E microscope (60× objective NA1.4) with Nikon Elements Software at 1 frame/3 s. In all, 488 and 561 nm lasers shuttered by Acousto-Optic Tunable Filters (AOTF) were used for the light source, and a custom quad-band excitation/dichroic/emission cube (based off Chroma, 89400) were applied for excitation and emission.

STEP-BY-STEP METHOD DETAILS

Live cell imaging in primary hippocampal neurons

© Timing: 6–12 h pretreatment + 2–6 h imaging time depending on number of replicates
Here we describe how to perform live imaging of MT dynamics at presynaptic boutons before and after pharmacological induction of neuronal activity.

1. Live cell imaging of EB3 comets in hippocampal neuronal culture is performed 24–72 h after transfection.
2. Prepare complete HBSS as recording medium and warm it up in a 37°C water bath.
3. For presynaptic MT dynamics we use an IX83 Andor Revolution XD Spinning Disk Confocal System with 37°C incubator and 5% CO₂. Stabilize the temperature and humidity of incubator before imaging. The microscope is equipped with a 100×/1.49 oil UApo objective, a multi-axis stage controller (ASI MS-2000), and a controlled temperature and CO₂ incubator.

**Note:** Axons are selected based on morphology (smaller diameter than dendrites and no spine structures along the neurite) and uniformly anterograde movement (toward the distal tip of the neurite) of EB3 comets. Proximal axonal segments (within 100 μm from the cell body according to our definition) are determined by first locating the cell body and then tracing the distance from cell body based on the real distance in μm in a still image taken with the camera.

4. Movies are acquired with an Andor iXon Ultra EMCCD camera and Andor iQ 3.6.2 live cell imaging software at 1 frame/3–4 s, 3 z-stacks at 0.4 μm step size for 3 min for dual channel acquisition.
5. For pharmacological induction of neuronal activity, neurons are pretreated with 50 μM D-AP5 for 6–12 h prior to live imaging in complete neurobasal medium, then changed to complete HBSS medium supplemented with 50 μM D-AP5. To induce neuronal activity, neurons are washed 3× with complete HBSS medium prior to addition of complete HBSS medium supplemented with 20 μM bicuculline or DMSO vehicle control. For BDNF induction of neuronal activity, 50 ng/mL BDNF is directly added to complete HBSS medium during live imaging.
6. Always acquire movies starting 1 min after induction of neuronal activity to ensure consistency among different experimental groups and to allow temperature and CO₂ flow to stabilize after addition of inducing medium.
7. Maximum intensity projections of movies are performed by Image Math within Andor software, exported as Tiff files, and analyzed in ImageJ.

**Live cell imaging in acute hippocampal slices**

© Timing: 2–3 h

Here we describe how to perform live imaging of MT dynamics at presynaptic boutons in acute hippocampal slices. This is based on the protocol from (Yasumatsu et al., 2008)

8. Acute slices are inspected under fluorescence illumination to identify the expressing regions of interest (CA1 and CA3) in the hippocampus.
9. Acute slices are transferred onto millicell membrane upside down in MatTek dishes with 1 mL of warm fresh sterile medium.
10. The MatTek dish is transferred in a temperature-controlled chamber attached to the microscope. Both temperature (37°C) and CO₂ flow (5%) are controlled and maintained to promote cell viability and optical stability during live imaging.
11. Electroporated hippocampal neurons from the CA1 region are selected and imaged using an inverted Nikon Ti-E microscope operated by Nikon Elements Software.
12. 488 and 561 nm lasers are used as light sources and shuttered by Acousto-Optic Tunable Filters (AOTF).
13. Live-imaging acquisition of EB3 comets relative to vGlut1 positive stable puncta within 50–100 μm of cell bodies is performed for 300 s using a 60× oil-immersion objective (NA1.4) at 1 frame/3 s.
EXPECTED OUTCOMES

EB3 comets and vGlut1/VAMP2 puncta can be observed in axons of primary hippocampal neurons in culture and acute hippocampal slices (Qu et al., 2019). In proximal axons (up to 100 \( \mu \)m from the cell body) of cultured hippocampal neurons EB3 comets move distally from the cell body while 72.3% of vGlut1 or 68.6% of VAMP2 puncta are stationary. An EB3 comet can appear in between (arrowhead) or right at (arrow) vGlut1/VAMP2 puncta and move along the axon until it falls off the MT plus end (Figure 2 and Methods Video S1 and S2).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Tiff files can be opened by ImageJ software. To better visualize the comets and reduce background, the average intensity projection image of a time-lapse can be subtracted from each frame. Click Image \( \rightarrow \) stacks \( \rightarrow \) z projects, then choose average intensity. The average intensity projection image of the movie will show up. Click process \( \rightarrow \) image calculator, then subtract the average intensity projection image from every frame of the movie. Save as a tiff file.

2. Kymographs are generated by drawing a region on the axon from the cell body to the most distal visible end of the neurite. Axons are identified based on morphology and uniform anterograde movement of EB3-labeled MT plus ends. Open the tiff file movie in MetaMorph software to generate kymographs. Draw a line along the neurite from the cell body to the most distal visible end of the axon. Click stack \( \rightarrow \) kymograph. Click measure \( \rightarrow \) calibrate distance to set pixel to \( \mu \)m conversion. Measure only comet tracks moving within 100 \( \mu \)m from the cell body. If doing the analysis in ImageJ, install KymoResliceWide plugin. Click Analyze \( \rightarrow \) set scale to convert px to \( \mu \)m. Draw the line along axons from cell the body to the end of the neurite. Normally axons need 10–15 px wide lines. Click plugin \( \rightarrow \) KymoResliceWide to generate a kymograph. Length of growth can be measured directly on the comet track as X-axis length. Comet lifetime is derived by the measured Y-axis length (Y \( \mu \)m) in the ratio of total kymograph width (D \( \mu \)m), which
represents the total length of movie time. For example, in a 180 s movie, comet lifetime (seconds) = 180 × Y/D. Then the growth rate can be calculated by growth length/comet lifetime.

3. Generate kymographs and distinguish between two groups of MTs, intrabouton and interbouton MTs as illustrated in the Graphical Abstract and in (Qu et al., 2019). Presynaptic MTs are classified based on their plus end contacts with stable vGlut1 or VAMP2 labeled boutons. Intrabouton MTs consist of MTs that nucleate/rescue at, catastrophe at, or pass through stable vGlut1 or VAMP2 labeled boutons. Interbouton MTs are those with no contact with vGlut1 or VAMP2 labeled boutons during the recording. In our measurements of EB3 tracks starting or ending at boutons, we also include those that start or end at a bouton and pass through the next distal or proximal boutons. Kymographs from both channels are overlaid to observe relative location of the comet tracks to stable boutons.

4. All MT dynamics parameters are defined as in (Qu et al., 2017; Qu et al., 2019; Stepanova et al., 2010) and calculated separately for intrabouton MTs and interbouton MTs.

Parameters describing MT dynamics are defined as follows:

| MT dynamics parameter       | Definition                                             |
|-----------------------------|--------------------------------------------------------|
| Rescue/nucleation frequency | number of rescue or nucleation events per μm² per min  |
| Catastrophe frequency       | number of full tracks/total duration of growth         |
| Comet density               | number of comets per μm² per min                        |
| Growth length               | comet movement length in μm                             |
| Comet lifetime              | duration of growth in seconds                           |
| Growth rate                 | growth length/comet lifetime                           |

5. Import data to GraphPad Prism and plot. If there are less than 15 axons per group or the dataset does not pass normality test, non-parametric tests will be used. Parametric/non-parametric T tests or two-way ANOVA are applied based on number of axons for all parameters. Averages of growth length, comet lifetime, and growth rate are calculated for each axon and statistical analysis is performed on those averages.

LIMITATIONS
The limitations of using primary neuronal cultures are predominantly associated with the challenge of maintaining healthy cultures for several weeks in vitro prior to transfection and the restrictions imposed by the embryonic nature of the cultures and loss of an intact circuit upon dissociation. The acute hippocampal slice approach is more laborious, and hence not well suited for drug screening or high throughput studies, and its success rate greatly depends on mouse survival from in utero electroporation.

TROUBLESHOOTING
Problem 1
Very few EB3 comets per axon or EB3 puncta do not appear to move in transfected neurons (steps 1–3).

Potential solution
This problem is typically caused by poor neuronal health after transfection or during live imaging. Be careful when changing to conditioned media and avoid creating shear stress on the neurons while pipetting. Plasmid DNA should be prepared using endotoxin-free maxi prep kits. Live imaging should always be performed in controlled and steady atmospheric conditions at 37°C with 5% CO₂, and over 90% humidity.

Problem 2
It is hard to visualize EB3 comets in the in utero electroporated acute brain slice if not used immediately after slicing (steps 5–7).
Potential solution
The duration between preparing the brain slices and live imaging should be kept at minimum. After cutting the slices, they should be immediately transferred into steady atmospheric conditions at 37°C with 5% CO2. These conditions must be maintained steady during the entire live-imaging session.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Francesca Bartolini (fb2131@columbia.edu)

Materials availability
This study did not generate unique materials or reagents.

Data and code availability
This study did not generate unique datasets or codes.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2021.100342.

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AUTHOR CONTRIBUTIONS
Conceptualization, X.Q. and F.B.; investigation, X.Q. and A.K.; writing – original draft, X.Q. and A.K.; writing – review & editing, X.Q., A.K., and F.B.; funding acquisition and supervision, F.B.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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