Live Imaging of MT dynamics in Dendritic Spines

1. Seed 60,000 neurons in the microwells of MatTek dishes (35 mm petri dish, 14 mm microwell). If you plate with less density (50,0000 neurons), the cells are easier to observe when the neurons are mature, but a less dense culture may be less tolerant of the transfection process.

2. Maintain hippocampal cultures to DIV 21. Healthy neuronal cultures are extremely critical.

3. To transfect neurons we use Lipofectamine 2000 reagents. For one dish, mix 0.5-2.5 ug of DNA with 300 ul of incomplete neurobasal medium (no B27, no antibiotics → critical for transfection efficiency). For co-transfection, mix the 2 DNAs together into incomplete neurobasal media at this step. Always titrate new DNA in the beginning to make sure cells are expressing right amount. For MT imaging in spines, we use 1.5 ug of EB3-EGFP (to visualize the comets) + 0.5 ug Tdtomato (the filler used to visualize the dendrites and spines) per dish.

4. For one imaging dish, add 3 ul of Lipofectamine 2000 to the diluted DNA mix. Incubate Lipofectamine-DNA mix at RT for 30 min.

5. Aspirate original conditioned media out from the dishes and save in a conical tube. Add lipofectamine-DNA mix dropwise to the microwells. Make sure the microwells are covered in liquid. Put the imaging dishes back to the incubator, and place the conditioned media in a 37ºC water bath.

6. Incubate for 2 hours.

7. Take the dishes out of the incubator, and aspirate out the transfecting reagents. Tilt the dish so that liquid is aspirated out completely and the pipette tip doesn't touch the cells. Add back 2 ml of conditioned media immediately. Do not let the cells dry.

8. Perform live imaging 40-48 hours later. Expression may not be strong enough 24 hours after transfection, and transfection can be toxic after 72 hours. Imaging is performed using a spinning disk confocal microscope at 100x magnification. Movies are taken at 4s/frame for 10 min and 3 z-planes with dual channel acquisition. A step size of .5um to .8um is usually sufficient to visualize all the spines.

- Imaging is performed in Complete HBSS (cHBSS).

Stock Solution	Volume	Final Concentration
10x HBSS	50 mL	1x
(no phenol red)		
1 M Hepes (pH7.4)	1.25 mL	2.5 mM
1 M D-glucose	15 mL	30 mM

- 100 mM CaCl2 5mL 1mM
- 100 mM MgSO4 5mL 1mM
- 1 M NaHCO3 2mL 4mM
- Add ddH2O to a total volume of 500 mL. Sterile filter with a 0.2 uM filter. Store at 4C.

9. Movies are analyzed by Fiji. For the EB3 channel, take the max intensity of the z stack, and then subtract the average intensity over time from the time course (to more easily visualize the comets). For the filler channel, take the max intensity of the z stack.

10. Spines analysis: Kymographs are generated by drawing a line from the base to the head of a spine. Parameters to analyze: length of growth (length of comet growing in the spine), comet lifetime (time of comet moving in the spine), growth speed (length of growth/comet lifetime), invasion lifetime (total time of a comet residing in the spine. You may see streaky vertical lines of EB3 on the kymographs which indicates the MT plus end is pausing. Include this time into invasion lifetime. With this technique (since there is no tubulin labeling), we do not analyze the time of shrinkage events, invasion frequency (number of MTs invaded into the spines/h), and % of MTs nucleated/rescued from local sites.