

## **LRRK2<sup>RCKW</sup> single molecule kinesin motility assays**

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### **Recommended Equipment and Setup:**

This single-molecule imaging experiment was originally performed using total internal reflection fluorescence (TIRF) microscopy with an inverted microscope (Nikon, Ti-E Eclipse) equipped with a 100x 1.49 N.A. oil immersion objective (Nikon, Plano Apo), and a MLC400B laser launch (Agilent), with 405 nm, 488 nm, 561 nm and 640 nm laser lines (561 and 640 nm laser lines are not needed for this version of the experiment). Excitation and emission paths were filtered using single bandpass filter cubes (Chroma), and emitted signals were detected with an electron multiplying CCD camera (Andor Technology, iXon Ultra 888). Illumination and image acquisition were controlled with NIS Elements Advanced Research software (Nikon), and the xy position of the stage was controlled with a ProScan linear motor stage controller (Prior).

### **Required Buffers:**

Streptavidin Buffer:

- 1 mg/mL Streptavidin
- 30 mM HEPES pH 7.4
- 2 mM MgOAc
- 1 mM EGTA
- 10% glycerol

Motility Assay Buffer:

- 30 mM HEPES pH 7.4
- 50 mM KOAc
- 2 mM MgOAc
- 1 mM EGTA
- 10% glycerol
- 1 mM DTT
- 20 uM Taxol

Motility Assay Buffer with casein:

- 30 mM HEPES pH 7.4
- 50 mM KOAc
- 2 mM MgOAc
- 1 mM EGTA
- 10% glycerol
- 1 mM DTT
- 20 uM Taxol
- 1 mg/mL casein

LRRK2 Buffer:

- 20 mM HEPES pH 7.4
- 80 mM NaCl
- 0.5 mM TCEP
- 5% glycerol
- 2.5 mM MgCl<sub>2</sub>

- 20  $\mu$ M GDP

5x BRB80 (only needed for initial microtubule assembly):

- 400 mM PIPES, pH 6.8 with KOH
- 10 mM MgCl<sub>2</sub>
- 5 mM EGTA

2x Polymerization Mix (only needed for initial microtubule assembly):

- 2x BRB80
- 2 mM DTT
- 2 mM GTP (Add last)
- 2 mM MgCl<sub>2</sub> (Add second to last)
- 20% DMSO
- Mix well between adding each ingredient

### Instructions:

1. Create taxol-stabilized microtubules (can be reused for multiple weeks):
  - 1.1. In a prechilled 1.5 mL Eppendorf tube, make a 10  $\mu$ L mixture of  $\sim$ 10 mg/mL tubulin. The mixture should be 80% unlabeled, 10% biotin-tubulin, and 10% 405-tubulin. Mix by gently flicking.
  - 1.2. Let it sit on ice for 10 min.
  - 1.3. Add equal volume of 2x polymerization buffer (10  $\mu$ L). Mix by gently flicking.
  - 1.4. Incubate at 37C for 30 mins. Make a 1x BRB80 + 1 mM Taxol + 20  $\mu$ M Taxol stock and incubate at 37C at the same time.
  - 1.5. Add equal volume of prewarmed 1x BRB80+DTT+Taxol (20  $\mu$ L). Mix by gently flicking.
  - 1.6. Incubate at 37C for at least 10 mins (solution will be stable for hours at this point).
  - 1.7. Store in the dark at RT. Should be usable for several weeks, but more aggregates will appear over time.
2. Create microscope slides:
  - 2.1. Adhere Biotin-PEG-functionalized coverslips (Microsurfaces) to a microscope slide using double-sided scotch tape, creating 4 channels per slide.
  - 2.2. Add the streptavidin buffer to each channel and incubate for 3 mins.
  - 2.3. Wash twice with Motility Assay buffer.
  - 2.4. Add a 1:150 dilution of taxol-stabilized microtubules (19  $\mu$ L per channel) and incubate for 3 mins.
  - 2.5. Wash twice with LRRK2 buffer. Add more buffer if necessary to prevent drying out.
3. Prepare LRRK2:
  - 3.1. Prepare a  $\sim$ 1  $\mu$ M solution of LRRK2<sup>RCKW</sup> in a cold LRRK2 buffer. Centrifuge through a 0.1  $\mu$ m PVDF filter to remove aggregates. Calculate the new effective concentration. Usually around 500-700 nM after centrifugation.
  - 3.2. Create a working aliquot of LRRK2 in the desired concentration (ex. 25-100 nM) in the LRRK2 buffer at room temperature (recommended volume of 25  $\mu$ L). If adding inhibitors, add them now with DMSO. Incubate for 10 minutes at room temperature.
4. Add LRRK2<sup>RCKW</sup> sample to the channel (19  $\mu$ L). Incubate for 5 minutes. Prepare step 5 while waiting.
5. Wash twice with the motility assay buffer supplemented with 1 mg/mL casein.
6. Prepare kinesin:
  - 6.1. Make a 4 nM solution of K560-GFP in the Motility Assay buffer with casein supplemented with an oxygen scavenger system ((0.4% glucose, 45  $\mu$ g/ml glucose catalase (Sigma-Aldrich), and 1.15 mg/ml glucose oxidase (Sigma-Aldrich)), 71.5 mM beta-mercaptoethanol and 1 mM ATP).
7. Add 19  $\mu$ L of kinesin mixture to each chamber
8. Image slide

### Imaging Recommendation:

Settings will vary per microscope.

We imaged K560-GFP every 500 msec for 2 mins with 25% laser (488) power at 150 ms exposure time. Each sample was imaged no longer than 15 mins. Each technical replicate recommended to consist of movies from at least two fields of view containing between 5 and 10 microtubules each.

Image using the 405 nm laser to determine the locations of the microtubules. Preferably at the start and end of the experiment.

**Single-molecule motility assay analysis recommendation:**

Kymographs were generated from motility movies and quantified for run lengths, percent motility, and velocity using ImageJ (NIH). Specifically, maximum-intensity projections were generated from time-lapse sequences to define the trajectory of particles on a single microtubule. The segmented line tool was used to trace the trajectories and map them onto the original video sequence, which was subsequently re-sliced to generate a kymograph. Motile and immotile events (> 1 sec) were manually traced. Bright aggregates, which were less than 5% of the population, were excluded from the analysis. Run length measurements were calculated from motile events only. For percent motility per microtubule measurements, motile events (> 1 sec and > 1 $\mu$ m) were divided by total events per kymograph. Velocity measurements were calculated from the inverse slopes of the motile event traces (> 1 sec and > 1 $\mu$ m) only. Statistical analyses were performed in Prism8 (Graphpad).