**31/10/2018 Carter lab TIRF microscopy Single Molecule Assay**

**5x Assay Buffer**

150 mM HEPES pH7.2, 25 mM MgSO4, 5 mM EGTA

**5x BRB80**

400 mM PIPES pH 6.8; 5 mM EGTA; 5 mM MgCl2

**Polymix (microtubule polymerization mixture)**

1 mL 5x BRB80, 1 mL 100% DMSO, 5.25 mg GTP - Final volume 5 mL with dH2O. 10 µl aliquots.

**DLB**

400 µL 5x Assay Buffer, 1600 µL H2O, 2 µL DTT (1 M)

**DLB-Casein**

400 µL 5x Buffer, 1500 µL H2O, 100 µL a-Casein (25 mg/ml *For preparation see below*), 20 mM KCl (or 25 mM), 2 µL DTT (1 M), 1 µL 100 µM Taxol *(Could try 10mM Taxol stock diluted 1:10 in water but MTs do start depolymerising)*

**GF150**

25 mM HEPES pH 7.2 (KOH)

150 mM KCl

1 mM MgCl2

**Microtubule Polymerisation**

On ice, in 1.5 ml Eppendorf tube mix:

+1 μL Alexa-647 (2 mg/ml)

+2uL Biotinylated Tubulin (2 mg/ml)

+6.5 μL Unlabeled tubulin (homemade porcine brain tubulin. *In this case 8.25 mg/mL. May need to adjust for different prep.*)

+0.5 μL BRB80 --- Mix all on ice

+10 μL 2x Polymix

Incubate at 37°C for 30-60 minutes. Cover in foil to protect from light.

*Carry out all subsequent steps at room temperature and with room temperature buffers.*

Add 100 μL BRB80-Taxol (2 μL 10 mM Taxol in 1 mL BRB80) gently to mixture

*NB: Do not pipette up and down to mix!*

Spin in benchtop Eppendorf centrifuge, 21K RCF, 8.5 mins. (Can often see MT pellet)

Pipette out supernatant, then add 100 μL BRB80-Taxol

Flick tube to mix

Spin 21K RCF, 8.5 mins

Pipette out supernatant, then add 50 μL BRB80-Taxol

*For good quality MTs for TIRF, do not flick to mix this time! Leave overnight at RT (protected from light)*

Next day, spin 21K RCF, 8.5 mins

Pipette out supernatant, then add 50 μL BRB80-Taxol

Flick to mix

Store for <1 week at RT protected from light

**Gloxy (glucose oxidase and catalase mixture)**

15 mg Glucose Oxidase + 2mg Catalase (both in -20) into 250 μL DLB

Make a 1 in 2 dilution in DLB and filter with 0.22 µM spin filter

After 3 hours or so, make a new dilution. Consider also spinning the 250 µL stock if background is still high

**Other Reagents Storage conditions**

45 % Glucose -80 °C

100 mM ATP -80 °C

*(Final ATP conc. is 5 mM, which is high. Could dilute. For preparation see below)*

Clean (HPLC-grade) BME – 1:1 in DLB (used to use 1:4) 4 °C

2 mg/mL PLL-PEG-Biotin – 1:3 in water -20 °C

1 mg/ml Streptavidin (NEB N7021S) 4 °C or -20 °C

**Reagents to filter before starting**

DLB, Water, PLL-PEG-Biotin

**Complex formation**

1 µL Dynein @ 0.4 mg/mL

1 µL Dynactin @ 0.4 mg/mL

1 µL BicD @ 0.8 mg/mL

Dilute protein stocks in GF150; 5 mM DTT; 0.1 mM ATP

Mix, leave on ice until needed for first slide (at least 15 minutes)

**Slide Preparation**

Piranha treated coverslips (see protocol below)

Make wide-ish chambers (want chamber vol to be ~10 μL).

For each wash, blot from the other end of the channel.

Set up chambers as below, so that imaging location is in center of slide:

*Typical set up for someone who pipettes with their right hand and blots with their left.*

Coverslip

Frosted end of slide

Slide

Blot from this side

Pipette stuff in here

Image around here

Double-sided tape (cut tape in half)

Flow in 10 μL PLL-PEG-Biotin

Flow in 5 x 10 μL DLB - wash from different points along the edge each time

Flow in 10 μL Streptavidin

Flow in 5 x 10 μL DLB

Filter 100-150uL DLB-Casein now

Dilute Microtubules in DLB-Casein (2 μL + 13 μL buffer is good place to start)

Try to take from same place in tube each time for consistency. Don’t pipette up and down. Could use a cut 20 μL tip for this.

Flow in 10 μL Microtubules, invert & incubate

Pipette in small droplet touching the front of edge of coverslip, then blot through quickly

*The longer you leave it, the more microtubules you will have, but the less parallel and straight they will be. 1 minute is a good starting point.*

Flow in 5 x 10 μL DLB

Flow in 5 x 10 μL DLB-Casein

Make reaction mix in order of addition; keep at RT – 14uL DLB-Casein, 2uL Complex (Diluted to 10uL), 1uL ATP, 1uL Gloxy, 1uL BME, 1uL Glucose

Flow in 2 x 10 μL DLB-Casein

Flow in 10 μL Reaction mix

**Imaging**

Image as close to the edge as possible, but ensure that oil drop does not touch edge of coverslip (see diagram).

*Usually MT and complex concentration need to be optimized on the day.*

**For data collection**

1 movie per slide, so ATP conc. isn’t depleted during the duration of imaging.

Clean objective between slides.

**100 mM Mg.ATP stock preparation**

**Reagents**

ATP (Sigma A- 2383, Disodium salt)

5 M NaOH

**Protocol**

Figure out the effective molecular weight after correcting for the water, ethanol, and purity.

Example

Formula weight: 551.1

+ 3 mol water: 551.1 + 3 x 18 =605.1

1% ethanol 605.1 x 0.01 = 6.051 +605.1 = 611.2

99% pure 611.2/0.99 = 617.3 g/ mole of ATP

2. Decide how much to make. 50 ml is a good amount. Using the above weight (may be different for different lot numbers):

 Weigh in 3.086 g NaATP

 + 1M MgAc 5ml

 + cold ddH2O 40 ml

 + 5 M NaOH 1500 µl

+ additional NaOH dropwise until pH reads 7 +/- 0.1 pH units.

Adjust volume to 50 ml.

For protein preps aliquot in 1-2ml fractions and store at -80.

For ATPase assays etc. you will require smaller (50ul) aliquots.

For the regular lab stock I usually use the above calculated MW (617.3g/mol). For more exact assays it might be necessary to redo the calculation.

**Casein preparation for motility assays**

**Reagents**

Casein (Sigma C-5890 stored at room temperature)

20 mM Tris pH 8.0

1 M NaOH

**Protocol**

Add 2g casein to 50ml of 20mM Tris pH 8.0

Check pH with indicator strips: probably about pH 7.0 – bring it up to pH 8.0 using NaOH

Leave on rotary wheel in cold room for ~1hour – check the pH again (probably about 6.0) – and adjust with NaOH to bring it back up to pH 8.0

Leave between 1 hour and overnight – check the pH again, adjust back to pH 8.0, and leave another hour if necessary.

Do a quick check of the protein concentration by Bradford (need it to be greater than 25 mg/ml) – Spin 50 µl of Casein solution in a microfuge, then take 1ul into 200ul Bradford, 800 µl water. If the solution is bright blue (OD595 is greater than 1.0) then the concentration is high enough.

Transfer Casein to Ti70 tubes and spin 65,000 rpm, 2 hours. Take off the clear middle layer (top layer is a little bit cloudy)

Do a Bradford with a BSA standard to get the protein concentration, dilute to 25mg/ml with 20mM Tris pH 8.0.

Filter through a 0.2 µm filter. Aliquot in 100 µl aliquots.

Snap freeze in liquid nitrogen and store at -80 °C.

Casein preparation is not as complicated as it looks from the above. The key is to get the Casein to dissolve to 30mg/ml. To do this you need to keep adjusting the pH back up to 8.0 using NaOH (Caesin is acidic and as it dissolves it lowers the pH).

**Piranha coverslip preparation**

***Method***

Rack up to 3 ceramic slide holders with 22x22mm slides.

Place into ~300 ml 3 M NaOH solution in a 1 L beaker (need enough to cover slides)

Sonicate in waterbath sonicator for 30 mins.

Wash extensively with MQ H20 (at least 6x beaker volume)

In fume hood, prepare Piranha solution in 1 L beaker ON ICE: 120 ml 30% H2O2 (in first), 180 ml sulfuric acid.

 Use glass measuring cylinders to measure both components

 Pour in sulfuric acid slowly, swirling the beaker in between pours. Wear proper PPE (including facemask, apron, not open shoes, check the rating of your gloves etc.)

Clean coverslips in Piranha under sonication for 30 mins. *Put warning sign on fume hood glass.*

Wash extensively with MilliQ H­2O (6x beaker volume)

Store under MilliQ water in a covered beaker.

Before making a side, take a coverslip and blow-dry using N2.

***Clean-up/disposal of chemicals***

Thoroughly rinse any glassware before leaving for the washup team!

Piranha solution will degrade relatively quickly over time. Leave for at least 2 h, then put whole beaker into a 5 L beaker of water, pre-filled with 3.5 L water first. Carefully carry out to sink, turn on taps to dilute piranha. Leave running until pH ~5 at least.

3 M NaOH - dilute into 3 L water in 5 L beaker (up to 1 L 3 M NaOH can be dealt with this way), add glacial acetic acid until pH is below 8 (in reality will get pH 4 or 5). Wash down sink with plenty water.

Drop spills of sulfuric acid or Piranha can be neutralised with powdered sodium bicarbonate (add quite a bit until doesn’t bubble anymore).