Flow Cell Assays with Microtubules: Motility/Dynamics in Fluorescence and VE-DIC

Flow cell assays are very useful for studying microtubule motility, microtubule dynamics, kinetochore-microtubule interactions and action of severing/depolymerizing factors on microtubules. Described here are some general procedures for flow cell assays.

I. Solutions & Supplies

BRB80 (1X): 80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8 with KOH (generally made as a 5X stock and stored at 4iC)

5 mg/ml casein (Sigma C-5890; dissolve at 10-20 mg/ml in 20 mM Tris, pH 8.0, on a rotator in coldroom for several hours; spin at 70K in TLA100.4 and collect clear middle layer. Measure protein concentration by Bradford using BSA as a standard and dilute to 5 mg/ml. This can be filtered if desired then aliquoted and stored at -20iC or -80iC)

10 mg/ml HSA or high purity BSA in 1XBRB80; store at -20iC or -80iC (HSA = Human Serum Albumin)

Oxygen Scavengers (see below)

Flow Cells: Flow cells can be constructed in many different ways. The most common way is to place two strips of double-stick tape on a glass slide ~7-10 mm apart and cover with a 18x18 or 22x22 mm coverslip. This results in a ~12-15 µl flow cell. Solutions are pipeted on one side and sucked out the other side by capillary action using Whatman #1 filter paper or a Kimwipe. Washes in flow cells should be ~4-8 chamber volumes and it is important to work in the middle of the cell (i.e. to avoid working close to the tape edge, where flow is not laminar resulting in poor washes/solution transfers).

For VE-DIC (video-enhanced differential interference contrast microscopy) coverslips must be cleaned thoroughly before use. We find that treating coverslips with acetone for 15'-30', followed by ethanol for 15', and then spin drying them works well for VE-DIC. Other labs use far more extensive and excruciating cleaning procedures. For fluorescence assays coverslips can be used straight from a box. In both cases, the coverslip surface is generally treated in

some way depending on the precise assay requirement, e.g., the surface is coated with casein prior to adsorbing motor proteins for motility assays. For fluorescence assays, oxygen scavenging is essential and a glucose oxidase/catalase/glucose system works very well for this purpose.

Axonemes (for dynamics assays)

II. VE-DIC Assays

A. Motility

Described here is a simple protocol for assaying kinesin motility by VE-DIC. Variations on this type of assay have been used to demonstrate motility of other MT motor proteins. This assay does not provide polarity information on the motility. Although there are methods for making polar microtubule substrates for DIC, we generally use fluorescent polarity marked taxol-stabilized microtubules to determine polarity, as described later.

1. Coat flow cell with a mixture of 0.25 mg/ml casein and kinesin (5-50 µg/ml) for 3'..

2. Rinse out unbound material with 80 µl BRB80 + 1 mM DTT + 1 mM MgATP + 10 µM taxol.

3. Transfer flow cell to microscope and setup DIC imaging.

6. Flow in 30 µl 10 ug/ml taxol-stabilized microtubules (unlabeled) sheared to ~5-10 µm length. The taxol-stabilized microtubules are diluted from a 1-2 mg/ml stock into BRB80 + 1 mM DTT + 1 mM MgATP + 1 mg/ml HSA + 10 µM taxol. After a reasonable density of microtubules have attached to the surface and started translocation, wash out unbound microtubules using 30 µl BRB80 + 1 mM DTT + 1 mM MgATP + 10 µM taxol (optional) and record motility. Once the reaction conditions are well-established, incubation with microtubules and removal of unbound microtubules can be done prior to transferring the flow cell to the microscope.

B. Dynamics

VE-DIC revolutionized analysis of microtubule dynamics by allowing observation of single dynamic microtubules in real-time. We have used VE-DIC for measuring dynamics of both pure tubulin and microtubules in clarified *Xenopus* egg extracts. We use axonemes as nucleators of microtubule assembly. The axonemes are adsorbed to the flow cell surface prior to flowing in tubulin. Dynamics measurements are generally performed in regimes where spontaneous nucleation is minimal -- thus, one can assume that the amount of polymer formed in the assay is miniscule and the monomer concentration is not changing during the observation. Care must be taken to ensure that this assumption is valid since depletion of monomer will affect the measured parameters of dynamics.

1. Coat a clean coverslip flow cell with axonemes and allow adsorption for 5' (adjust concentration and time of adsorption such that density is ~1-3 per field-of-view on the monitor).

2. Wash out unbound axonemes with 80 µl of BRB80 + 1 mM DTT + 1 mM GTP + 0.5 mg/ml HSA

3. Flow in at least 3-4 chamber volumes of tubulin mix (i.e. tubulin in BRB80 + 1 mM DTT + 1 mM GTP + 0.5 mg/ml HSA), seal edges with Valap (1:1:1 mix of vaseline:lanolin:paraffin) and begin observation. To avoid surface effects, dynamics are measured by following microtubule ends growing down into the flow cell away from the coverslip surface -- these ends exhibit brownian motion in a focal plane below the coverslips surface as they polymerize/depolymerize.

For mammalian tubulins, the temperature is raised to 35-37iC during observation. We have done this using warm air blowers or by wrapping the high NA condenser and objectives with thin silicone tubing through which hot water is circulated -- the temperature of the water is empirically adjusted such that the temperature of the immersion oil on the coverslip surface is 35-37iC (measured using a thermocouple probe). A variety of home-rigged schemes for controlling temperature on the microscope stage have been developed in different labs.

For assaying dynamics of microtubules in *Xenopus* egg extracts, we simply flow ~50 µl of extract through a flow cell with adsorbed axonemes and then seal the cell with Valap. The high protein concentration of the extract eliminates need for blocking the coverslip

surface.

III. Fluorescence Assays

A. Oxygen Scavenging:

For live fluorescence microscopy it is essential to scavenge oxygen in order to limit photodamage. The most convenient method for doing this is using a glucose oxidase/glucose/catalase mix (OS mix). The component enzymes are stored as 100X stocks at -80iC and thawed and used for ~2 hours after mixing. It is important to keep the OS mix in a sealed tube on ice. The principle by which the mix scavenges oxygen is as follows:

catalase: $H_2O + O_2$------->H_2O_2

glucose oxidase: D-glucose + H_2O_2 ------->D-glucono-1,4-lactone

A1. 100X Stock Solutions:

Glucose: 450 mg/ml in ddH_2O (~ 2.25 M)

2-mercaptoethanol: 50% (~ 7.15M; dilute from stock before use)

Glucose Oxidase: 20 mg/ml (Sigma G-2133)

Catalase: 3.5 mg/ml (Sigma C-40)

The glucose oxidase and catalase are made up in 12 mM K-PIPES, pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA, frozen in 20-50 ul aliquots in liquid nitrogen and stored at -80iC. We find it most convenient to prepare the glucose stock along with the glucose oxidase and catalase stocks, and store all three stock solutions in different colored tubes at -80iC.

A2. Using OS Mix:

1. Thaw 100X stocks of glucose, glucose oxidase and catalase and store on ice. Prepare a 50% (v/v) 2-mercaptoethanol stock solution on ice.

2. Make a 10X OS Mix on ice: (the following recipe is for 50 µl)

30 µl BRB80 (0iC)

5 µl of glucose oxidase, catalase, 2-mercaptoethanol and glucose stocks

Add glucose last, after mixing the other components, and store the 10X OS Mix in a sealed tube on ice (i.e. do not leave top open). Keep the tube on ice and ensure that temperature of solution to which the OS Mix is added only increases and does not decrease. Prepare a fresh 10X stock after ~2 hours for optimal results. Add 1/9 vol of the 10X OS to the solutions used for washing flow cells so that the sample is well equilibrated with 1X OS mix prior to observation. Using this recipe, kinesin motility can be recorded with an unshuttered/unattenuated mercury arc for >15'.

B. Assays

Motility assays in fluorescence are generally performed with polarity marked taxol-stabilized microtubules (see page 187) to determine the polarity of the assayed motor protein. Kinetochore-microtubule interaction, microtubule severing and microtubule depolymerization assays have also relied heavily on fluorescence microscopy combined with flow cell technology. It is important to be aware that fluorescence assays are subject to photodamage artifacts, especially if oxygen scavenging is not done properly, and also that the oxygen scavenging mix may affect the property being assayed (this is a real concern for measuring microtubule dynamics using fluorescence). Described below is a kinesin motility assay with polarity marked taxol microtubules.

1. Coat flow cell with a mixture of 0.25 mg/ml casein and kinesin (5-50 µg/ml) for 3'.

2. Rinse out unbound material with 80 µl BRB80 + 1 mM DTT + 1 mM MgATP + 10 µM taxol + 1X OS Mix.

3. Flow in 30-50 µl of 10 ug/ml polarity marked taxol microtubules diluted from a 1-2 mg/ml stock into BRB80 + 1 mM DTT + 3 mM MgATP + 10 µM taxol + 1X OS Mix. Focus on the coverslip surface on an upright epifluorescence microscope.

4. After a reasonable density of microtubules have attached to the surface and started translocation, wash out unbound microtubules with 30 µl BRB80 + 1 mM DTT + 3 mM MgATP + 10 µM taxol + 1X OS Mix (optional). Focus on a field-of view on the coverslip surface near the middle of the flow cell and record motility using shuttered attenuated illumination and a digital or video camera.

Although good oxygen scavenging will allow observation with unshuttered illumination, it is a good idea for any live fluorescence studies to use shuttered and attenuated illumination (attenuation is generally done using neutral density filters). It is also essential to keep an eye open for a decline in the performance of the OS Mix. If bleaching becomes apparent when focusing on the sample, prepare a fresh 10X OS Mix from stocks stored at -80iC.