Single-Molecule Restriction Enzyme Digest Using Tethered Particle Motion (2012)

In this experiment we will be watching the restriction enzyme HindIII cut single molecules of DNA by attaching one end of the DNA to a microscope coverslip and the other end of the DNA to a micron-scale bead whose motion we can monitor under the microscope. When the enzyme cuts the DNA tethering the bead near the surface, the bead should diffuse away into solution. By the end of this experiment you should be able to calculate the rate of the enzyme's cutting activity by analyzing the rate at which beads leave the field of view.

This experiment requires a delicate touch so work carefully and don't rush!

DNA tethering protocol

1) We have prepared for you flow chambers that consist of a channel formed between two pieces of double-sided tape, a coverslip, and a slide. You will pull liquid through the chamber by continuously pipetting into the side with the tape overhangs while using a Kimwipe to pull the liquid out the other side. **Don't get air bubbles in the chamber!**

Place the chamber coverslip-side-down on the bench so that you can pipette into the tape overhang channel.

2) Pipette 50 µL of anti-digoxigenin into the channel and pull it through with a Kimwipe. **Leave a drop on each end of the channel so that it doesn't dry out!** Incubate for 10 minutes.

Anti-digoxigenin is an antibody that binds a small molecule called digoxigenin, which has been attached to one end of the DNA that you will use. Anti-dig sticks nonspecifically to glass.

3) Wash out excess anti-dig by pulling 500 µL of the "P" buffer through the channel.

4) Wash again by pulling 500 µL of the "3P" buffer through the channel.

5) Pull all 250 µL of DNA into the chamber, again leaving a drop on each end of the chamber (or adding drops with extra DNA or buffer) so that the chamber doesn't dry out. Incubate for 20 minutes.

This DNA is 900 bp long and has a single site for the restriction enzyme HindIII roughly in the middle. It has a different small molecule on each end: on one end is the digoxigenin that will bind to the anti-dig on the surface of the coverslip, and on the other is a biotin molecule that will attach to the streptavidin molecules on the beads.

6) **During the DNA incubation**: Prepare the beads by washing them three times as below. This exchanges the buffer that they're stored in for the buffer we use in the experiment, plus it removes any free streptavidin in the storage solution that has come off the beads, and allows us to coat the beads with a surface blocker called casein that's in the 3P buffer, which

prevents the beads from sticking to the surface or the DNA. (Note that this blocking agent isn't perfect—you'll still see beads stuck to the surface under the microscope!)

To wash the beads:

a) Add 24 µL of 3P to 6 µL of beads.
b) Spin at 13,000 rpm for 3 minutes to pellet the beads.
c) **Gently** remove the supernatant without disturbing the pelleted beads.
d) **Gently** resuspend the bead pellet in 30 µL 3P, without getting bubbles!
e) Spin again, remove supernatant and resuspend in 30 µL.
f) Spin a third time; this last time resuspend the beads in 50 µL 3P.

7) After the DNA incubation, wash out unbound DNA from the channel with 500 µL 3P.

8) Pull most of the 50 µL of beads into the chamber, leaving just enough so that you can add extra bead liquid to either end so the chamber doesn't dry out. Incubate for 10 minutes.

9) Wash out excess beads with 500 µL 3P.

Digest

10) Set up the sample on the microscope: use a 100x objective and brightfield microscopy (no phase). Find a nice field of view with ~30 beads, if possible. Focus the beads so that they're black spots with a ring around them. **It's easiest to focus on the tape first and then move into the channel and try to find the beads.** The beads will be lower in z than the tape. Under the microscope the tape looks like criss-crossing threads.

11) **CAREFULLY** pipette 300 µL of NEB2 buffer onto the input-end of the channel, without bumping the slide. (It's easiest to drop the buffer onto the coverslip, without touching the pipette tip to the coverslip.) Carefully use a Kimwipe to pull the liquid through the channel, again without bumping the sample!

This NEB2 buffer is the same as you would use for a normal, bulk HindIII digest in a test tube, except that the surface blocker casein has been added to keep the beads from sticking to the surface.

12) Take a control movie for 5 minutes, at about 30-60 seconds per frame. At most only a couple beads should dissociate.

13) Carefully, as you did before, add 200 µL of NEB2 plus HindIII to the input-end of the channel, and pull it through the chamber. You might want to try to take a movie while this is happening, if you can avoid bumping the sample while pulling the enzyme into the chamber.

The TA's added 2-4 µL of HindIII enzyme stock to the 200 µL that we give you.

14) Take a 5 minute movie (or continue the movie you started during the flow-through of the enzyme.) At the end of 5 minutes all but a couple beads should be gone!