Submission ID #: 61236
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18677028

Title: Parallel High Throughput Single Molecule Kinetic Assay for Site-Specific DNA Cleavage

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **YES**

   If **Yes**, can you record movies/images using your own microscope camera?

   YES

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Emily Matozel:** This protocol can be applied to any process that results in double stranded DNA breaks. It yields detailed, quantitative data on kinetics.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Emily Matozel:** Although this method has single molecule resolution, it measures up to one thousand DNAs in a single experiment, thus providing good statistics.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Emily Matozel:** The primary application of this method is to study the mechanisms involved in DNA binding and modification. For example, we are interested in DNA target search, which is relevant to all DNA binding proteins.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Nathaniel Dale:** When attempting this technique for the first time, remember that single molecule tethers are delicate, and once formed must be handled with care. Proper preparation of functionalized surfaces is essential.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Nathaniel Dale:** There are a few tricks for making the flow cell, as well as switching sample tubes during data collection. Visual demonstration can help someone who is new to this procedure.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Making the Flow Cell

2.1. To wash the coverslips, place them in staining jars **[1]** and sonicate them with ethanol for 30 minutes **[2]**. Thoroughly rinse them with deionized and distilled water **[3]**, then immerse them in 1 molar potassium hydroxide and sonicate them for 30 more minutes **[4]**.

2.1.1. WIDE: Establishing shot of talent at the lab bench placing coverslips in a staining jar.

2.1.2. Talent sonicating the coverslips. *Videographer: Obtain multiple usable takes because this shot will be reused in 2.2.1.*

2.1.3. Talent rinsing the coverslips with water.

2.1.4. Talent filling staining jar with KOH, with the KOH container in the shot and labeled.

2.2. Repeat the wash sequence, making sure to rinse the coverslips with water after each wash **[1]**. Store the cleaned coverslips in staining jars with water **[2]**.

2.2.1. *Use 2.1.2.*

2.2.2. Talent filling staining jar with water.

2.3. Use a clean razor to cut 8-centimeter long loading and exit tubes **[1-TXT]** and insert them into holes in a clean glass slide **[2]**. Epoxy the tubing for 5 minutes to secure it **[3]** and trim off any excess **[4]**. *Videographer: This step is important!*

2.3.1. Talent cutting tubing. **TEXT: PE-20 for inlet ; PE-60 for outlet**

2.3.2. Talent inserting the tubing into holes.

2.3.3. Talent adding epoxy to secure the tubing.

2.3.4. Talent trimming the tubing.

2.4. Apply double-sided tape precut with the channel pattern to the glass slides **[1]** and smooth it out with plastic forceps to achieve a good seal **[2]**. Peel the backing off the tape **[3]** and apply a clean coverslip, smoothing it out with the forceps **[4]**. Then, epoxy the edge of the coverslip to seal the flow cell and let it cure **[5]**. *Videographer: This step is important!*

2.4.1. Talent applying the tape to a slide.

2.4.2. Talent smoothing the tape.

2.4.3. Talent peeling off the backing from the tape.

2.4.4. Talent applying a coverslip and smoothing it with forceps.

2.4.5. Talent applying epoxy to the edge of the coverslip.

3. Tethering of DNA and Beads

3.1. Prepare the labeled DNA for tethering by performing PCR according to manuscript directions, then purify the PCR product with a PCR clean-up kit **[1]**.

3.1.1. Talent putting tubes in a thermocycler and closing the lid, then programming the thermocycler.

3.2. Prepare 10 milliliters of Buffer A and degas it in a vacuum desiccator for at least 1 hour **[1-TXT]**. To functionalize the flow cell, inject 25 microliters of anti-digoxigenin FAB *(pronounce 'fab')* fragments in PBS into the flow channel, using a gel loading tip to fit into the PE-60 tubing **[2-TXT]**. Incubate the flow cell at room temperature for 30 minutes **[3]**.

3.2.1. Talent degassing Buffer A. **TEXT: 1 M Tris-HCl [pH = 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 1 mg/mL β-Casein, 1 mg/mL Pluronic F-127**

3.2.2. Talent injecting anti-digoxigenin FAB fragments into the flow channel. **TEXT: 20 µg/mL**

3.2.3. Flow cell at room temperature.

3.3. After the incubation, pull 0.5 milliliters of buffer A through the channel with a syringe, taking care to not introduce air into the channel **[1]**. Then, mount the flow cell on an inverted microscope **[2]**, hook up the outlet tube to a syringe pump **[3]**, and put the inlet tube into a microcentrifuge tube containing buffer A **[4]**. *Videographer: This step is important!*

3.3.1. Talent pulling buffer A through the channel.

3.3.2. Talent mounting the flow cell on the inverted microscope.

3.3.3. Talent hooking up the outlet tube to the syringe pump.

3.3.4. Talent putting the inlet tube into a microcentrifuge tube with buffer A, with the tube labeled if possible.

3.4. Manually pull at least 0.5 milliliters of buffer A to flush the system and prime the pump **[1]**, then let the pump run at 10 microliters per minute for at least 5 minutes to equilibrate the system **[2]**.

3.4.1. Talent manually flushing the system with buffer A.

3.4.2. Talent switching the pump on.

3.5. Vortex the stock bottle of beads **[1-TXT]** and pipet 1.6 microliters of the beads into 50 microliters of buffer A, then vortex them again **[2]**. Place the beads on a magnetic

separator and pipet out the buffer **[3]**, resuspend them in 50 microliters of buffer A, and vortex them **[4-TXT]**.

3.5.1. Talent vortexing the bead stock bottle. **TEXT: 10 mg/mL**

3.5.2. Talent adding beads to a tube with buffer A.

3.5.3. Talent pipetting the buffer from the beads on a magnetic separator.

3.5.4. Talent resuspending the beads. **TEXT: Repeat for a total of 3 washes**

3.6. After the last wash, resuspend the beads in 100 microliters of buffer A for a final concentration of 160 micrograms per milliliter **[1]**. Prepare 480 microliters of 0.5 picomolar labeled DNA substrate in buffer A **[2]** and pipette 20 microliters of bead suspension into the diluted DNA **[3-TXT]**. Place the mixture on a rotator for 3 minutes **[4]**.

3.6.1. Talent resuspending the beads in buffer A.

3.6.2. Talent diluting the DNA in buffer A.

3.6.3. Talent adding beads to the tube with the DNA. **TEXT: Make sure beads are vortexed!**

3.6.4. Sample tube on the rotator.

3.7. After the 3 minutes, immediately load the sample into the channel at a flow rate of 10 microliters per minute **[1]** for 15 minutes or until sufficient bead tethering is observed **[2]**.

3.7.1. Talent inserting inlet tubing into sample tube and turning on pump to load the sample into the channel.

3.7.2. LAB MEDIA: 3-7-2_loadBeads.wmv. Microscope video of beads loading into channel.

3.8. Wash the channel of all free beads by switching the inlet tube to a fresh tube of buffer A and flowing it in at 50 microliters per minute **[1]** for at least 10 minutes or until no loose beads are observed **[2]**. *Videographer: This step is difficult!*

3.8.1. Talent switching the inlet tube to a fresh tube of buffer A, with the tube labeled and restarting pump

3.8.2. LAB MEDIA: 3-8-2_endOfWash.gif. Microscope video showing few loose beads near end of wash.

3.9. **Nathaniel Dale**: It is helpful to use an inline valve to cutoff flow when switching the tubing. Switch tubing in one smooth motion and make sure liquid levels in new and old sample tubes match to avoid backflow.

3.9.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

3.10. Before collecting data, prepare the correct concentration of DNA cleavage enzyme and insert the inlet tube into the sample **[1]**. Set the pump to inject 80 microliters of sample at 150 microliters per minute **[2C]**, lower the magnet over the flow cell **[2]**, and begin data collection **[2B]**. One minute into data collection, activate the pump **[3]**. *Videographer: This step is difficult and important!*

3.10.1. Talent inserting inlet line into sample tube.

3.10.2. Talent lowering magnet over sample

3.10.2B Added shot: Talent starting data collection

3.10.2C Added shot: Talent activating pump

3.10.3. LAB MEDIA: 3-10-3_dataCollection.gif. Microscope video showing what is recorded

Results

4. Results: NdeI Cleavage is Dependent on Concentrations of Protein and Magnesium

4.1. Shown here is a representative image of the tethered beads before and after the reaction **[1]**. Data analysis was performed to quantify the number of beads remaining tethered throughout the cleavage reaction **[2]**.

4.1.1. LAB MEDIA: Figure 3 A and B. *Video Editor: Label A "***Before cleavage***" and B "***After cleavage***".*

4.1.2. LAB MEDIA: Figure 3 C. *Video Editor: Label the green area in the graph "***Enzyme Injection***".*

4.2. This technique was used to measure the site-specific DNA cleavage rates of NdeI *(pronounce 'N-D-E-one')* for protein concentrations ranging from 0.25 to 4 units per milliliter and two different concentrations of magnesium. Each condition was replicated at least twice, with a few hundred to 1,000 tethered DNAs per experiment **[1]**.

4.2.1. LAB MEDIA: Figure 4.

4.3. At sufficiently low protein concentrations, the rate is proportional to protein and independent of magnesium **[1]**. For high protein concentrations, the rate is dependent on magnesium but independent of protein concentration **[2]**.

4.3.1. LAB MEDIA: Figure 4. *Video Editor: Emphasize the first 2 data points on both lines.*

4.3.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the last 2 data points on both lines.*

Conclusion

5. Conclusion Interview Statements

5.1. **Allen C. Price:** When attempting this protocol, keep in mind that air bubbles in the tubing and flow channel can ruin the experiment. Make sure not to allow any air into the lines while switching tubing and degas all buffers before using.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.8.1 and 3.2.1.*

5.2. **Allen C. Price:** The tethering method allows one to explore the effect of tension in the DNA on the reaction. This can be achieved by varying the magnet strength or applying flow during data collection.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.