Single-molecule DNA barcoding in nanofluidic channels

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Introduction
In this experiment the student will take a “barcode” of a single DNA molecule from the genome of the λ-phage virus. This barcode is a unique, coarse-grained description of the base-pair sequence. Although the resolution of this barcode to ~1000 base pairs by diffraction, in comparison to a full base-pair sequence a barcode can be obtained very rapidly and with a simple set of reagents. As well, because it can be done to single molecules, this barcoding technique can detect genomic differences between cells that are invisible to conventional sequencing which requires averaging over many DNA molecules. Barcoding processes like this one may make it possible to study the genomic heterogeneity between cancer cells that is thought to confer drug resistance.¹

The student will perform a kind of barcoding developed by Walter Reisner called denaturation mapping.² In the context of DNA, denaturation refers to the process of dissociating from double to single strands. Denaturation mapping makes use of a handful of ideas to produce a barcode:

- The probability that a DNA molecule will denature depends on its sequence, because G-C base pairs have a higher bonding energy than A-T pairs.
- In a solution of 50% formamide, a typical DNA molecule will begin to denature at ~27°C.
- Whether DNA is denatured or not can be revealed by staining it with the fluorescent label YOYO-1. YOYO-1 binds to double-stranded but not single-stranded DNA.
- While a DNA molecule coils into globular shape, DNA confined to sufficiently small channels (width ~100nm) is forced to stretch into a linear conformation. For DNA stretched this way, position along the channel is linearly related to position along its sequence.

Consequently, gently heating a DNA molecule dyed with YOYO-1 in a formamide solution while confined to a nanochannel produces a linear “barcode” of fluorescence that characterizes denaturation as a function of position along the DNA sequence. This barcode can be used to identify the location of a single DNA fragment on the genome by aligning it to a computationally generated barcode of the entire genome made from a theoretical model.

The student will reproduce a Reisner’s proof-of-concept experiment (Reisner 2009) to test the denaturation mapping technique. In broad terms the experiment involves the following steps: loading previously prepared DNA into a nanofluidic chip; driving DNA to the nanochannels using a pump; partially denaturing the DNA using a heating element; and recording images of the fluorescence pattern of the DNA molecule. Analyzing these images involves two computational steps using previously-developed MATLAB algorithms: time-averaging to produce a normalized barcode and comparing the barcode to a previously computed one in order to align the fragment to the genome. A successful result (confident alignment of the experimental and computational denaturation maps) would demonstrate the usefulness of denaturation mapping as a single-molecule barcoding technique.

This experiment will be performed at Walter Reisner’s nanobiophysics lab in Rutherford 441.

Experiment

1. Introduction to apparatus

Denaturation mapping is done using an apparatus made of connected parts on the nano, micro and macroscales. On the smallest scale, the nanochannels where barcoding occurs are part of a fluid circuit referred to as a nanofluidic “chip.” A nanofluidic chip is to a plumbing system what an electronic chip is to a tabletop circuit – a miniaturized version that is fabricated on a wafer. This experiment’s chip is made from a 2.5cm square of fused silica wafer sealed with a silica coverslip. The array of nanochannels (width 120nm) at the centre of the chip connects to two larger microchannels (width 50μm), that in end in macro-scale reservoirs (radius 1mm).

Figure 1: The nanofluidic chip. The lines running to the centre of the chip are microchannels. The array of nanochannel are where the microchannels appear to intersect. They’re too small to see by eye.

The part that connects the chip to the apparatus’s macroscale components – air pump, the heater and the microscope – is called a chuck. The chip sits on the chuck which sits on the microscope. The chuck is made from a 7cm wide piece of machined plastic. The air pump connected to it control flow in the chip, the heater mounted on it controls the temperature in the chip, and the microscope under it images DNA during the experiment.

Figure 2: The chuck mounted on the microscope with the pump and heater attached. From (Reisner 2009).
Performing denaturation mapping is fast. A good barcode can be made from images taken over the course of 1 minute. However, because the apparatus has many parts that must be put in place carefully, setting up the chip to do denaturation mapping takes time. You should set aside at least an hour and a half to set up this experiment before taking data.

Figure 3: A schematic of the chuck, chip and heater as they fit together. From (Reisner 2009).

Outline of set-up procedures in the following section:
- Loading the chip
- Mounting the chip on the chuck
  - Connecting the air pump
  - Mounting the heater
- Mounting the chuck on the microscope
  - Mounting the chuck on the chuck holder
  - Mounting the chuck holder on the microscope
- Setting up imaging
  - Moving the chip in place
  - Finding nanochannels
  - Confining DNA to nanochannels
2. Set-up

*Loading the chip*

The \(\lambda\)-phage DNA for this experiment has been stained with YOYO-1 and prepared in a buffer. This buffer, 0.05XTBE + 10mM NaCl, stabilizes the pH of the solution surrounding the DNA. As well it contains 50\% formamide to help denature DNA. Finally, it also includes the reagent BME and oxygen-scavenging enzymes glucose oxidase and calyase that help prevent *photonicicking*, the process by which fluorescence of YOYO-1 leads to DNA strands breaking. Because these enzymes are sensitive to temperature the buffers are kept on ice until they are used. BME and formamide are extremely toxic and care should be taken to avoid exposure to them. Wear a lab coat and gloves for the entire experiment.

There are two buffers prepared, one with DNA and one without. The oxygen-scavenging enzymes require glucose to function. This glucose is added just before the experiment to preserve the functionality of the enzymes.

- Put on a lab coat and nitrile gloves.
- Pipet 1\(\mu\)l of beta-D glucose into both the DNA and no-DNA buffers.

The chip is kept submerged in buffer so that it doesn’t dry out. This prevents contaminants from being deposited and bubbles from forming.

- Using tweezers, remove the chip from its container. Wipe gently with a kimwipe to remove big drops of buffer.

![Figure 4: A schematic of the fluidic reservoirs, microchannels and nanochannels on the chip. The maroon circles are reservoirs, the maroon lines are microchannels, and the black lines running between the maroon lines are nanochannels. Four reservoirs are numbered as described in the experimental set-up below. The other four reservoirs, as well as the green rectangle, are features that aren’t used for this experiment.](image-url)
- Place the chip on a clean absorbing paper with the reservoirs face-up, so that the reservoirs leading to the nanochannels are at the top and bottom of the chip, as in Figure 4 (call these 1, 2 in the top row and 3,4 in the bottom row, counting left to right). Use the two rectangular stripes that diffract brightly in the centre of the chip was a guide: these should be on the left and right side of the chip’s centre.

- Pipet 2 μl of DNA buffer into reservoirs 1 and 4, touching the tip directly to the reservoir and tapping out any bubbles that form. Try and work quickly so the unfilled reservoirs don’t dry out.

- Pipet 2μl of non-DNA buffer into reservoirs 2 and 3, and the other four that don’t connect to the nanochannels.

**Mounting the chip on the chuck**

The chuck’s largest face has ports that match the chip’s reservoirs. These labeled in the same numbering scheme that I’ve used here for the chip.

![Figure 5: The chuck, “top side” (left) and “underside” (right). On the top side, the smaller numbers label the ports to connect to the chip larger numbers label the luer ports to connect to the air pump. On the underside, the chip sits in the indented square. The descriptions in the following section make use of the directions “up” and “down” denoted by the black arrows in the left image.](image)

- Flip the chuck horizontally so the unlabelled underside is face-up on the bench. Reservoirs 1,2 should be still pointing up and 3,4 still pointing down.

- Holding the chip, flip it horizontally in the same way you flipped the chuck and place it on the indented square in the middle of the chuck. Nudge the chip carefully using tweezers so that the reservoirs line up with the ports on the chuck. Be careful that the sides of the chip don’t extend past the indented square.

- Place the square metal retaining ring over the chip so that the four holes in its corners line up with screw-holes in the chuck. Using tweezers, place the screws in the holes without disturbing the chip.
• Gently turn the screws with an allen key until the screws begin to give resistance. Using with the larger arm of the allen key, turn each screw about a quarter-turn more, until it gives resistance that matches what you can exert easily with one finger. The o-rings on the chuck’s ports should be visibly compressed against the chip in order to create a seal. Be very careful not to exert too much force because the metal bracket can crack the chip.

• Flip the chuck horizontally so that the top side is face-up again.

• Seal the top of the chuck’s eight ports using screws fitted with o-rings. You can screw these tightly because they don’t touch the chip. This seal should be airtight.

**Mounting the chuck on the microscope**

Once the chip is mounted on the chuck, you’re ready to connect the chuck to the apparatus’s macroscopic components for manipulating DNA, the air pump and heater. It’s easiest to put these in place before the chuck is placed on the microscope.

• Bring the chuck to the optical table. Put it on a clean absorbing paper so that you don’t lose any small parts through the screw-holes in the optical table.

• **Connecting the air pump**
The air pump feeds four lines that end in numbered luer locks. Each of these lines is controlled by a numbered switch in front of the microscope, and the four in total are controlled by a master switch. The “up” position opens a line and the “down” closes it.

  o Screw the each of the lines’ luer locks into the corresponding luer ports on the side of the chuck. Make sure the numbers match.

  o It’s important to check before moving on that the connections between the pump and the reservoirs on the chip are sealed. Turn on the pump and the pump meter. Open the switch for port 1 and close the rest. Using the master switch, try to exert about 500mbar of pressure. Hold the chuck to your ear and listen for leaks from the chip. If the pump succeeds in exerting the pressure without producing a hiss, port 1 is sealed. Repeat this check for ports 2, 3 and 4.

    ▪ If a port hisses, very gently tighten the screw on the square metal retaining ring closest to that port and test it again. If the o-rings are visibly compressed the port should seal.

• **Mounting the heater**

  o Using a loose pipet, apply a layer of thermal grease to the heating element. The grease should form a thin but complete coating.

  o Insert the heater into the hole in the centre of the chuck, stopping when you feel it contact the chip. Rotate the heater back and forth to spread the thermal grease for good thermal contact.
- **Mount the heater on the chuck holder**
  A plastic holder keeps the chuck in place on top of the microscope. It has been machined to match the dimensions of the microscope stage.
  - Place the chuck on the holder so that the pump lines extend across the long, horizontal dimension of the holder. Insert long screws through the four holes on the outside of the chuck, and then through the corresponding holes on the chuck holder, and fasten them by hand using nuts.

- **Mounting the chuck holder on the microscope**
  - Use the microscope’s rough focus adjustment knob to make sure the lens is backed as far down as possible to avoid bumping it with the chuck.
  - Carefully place 1 drop of immersion oil on the lens. Using the Bertrand lens on the eyepiece, check there are no bubbles in the oil. If there is a bubble, wipe the gently lens using isopropyl alcohol on the corner of a kimwipe and try again.
  - Place the chuck holder on the microscope stage, once more so that the pump lines extend horizontally.

**Setting up imaging**
Often the most difficult task in setting up the microscope to image DNA in nanochannels is to find the nanochannels. Because they are so small, when the chip is first brought into focus they are almost never in the field of view. Using the positioning stage on the microscope you’ll need to scan the chip to find the larger microchannels and then follow these to the nanochannels.

- **Moving the chip in place**
  - Direct the optical path to the eyepiece (marked EYE) and set the microscope mirror to point to the incandescent epi lamp (marked M). Turn on the epi-mode incandescent lamp (labeled EPI) to maximum intensity. Also, turn on the PRIOR positioning stage and Sutter shutter on the same rack. Make sure the density filters ND4 and ND8 are out and that the field and condenser apertures are all fully open so that the lamp shines with full intensity. A yellow light should shine from the objective onto the chip.
  - Watching the objective and the underside of the chip from next to the microscope, slowly adjust the focus to bring the objective close to the chip. Once it is close use the joystick for the microscope’s positioning stage to roughly centre the objective on the chip.
  - Still looking at the chip, slowly adjust the microscope focus until the bead of oil on the objective makes contact with the chip. Be very careful not to move the lens too far. The lens is valuable and can be scratched easily by crashing into the chip.
  - **Adjusting the microscope focus to scan for microchannels**
- Close the field aperture halfway. Looking through the eyepiece, adjust the fine microscope focus to slowly bring the objective closer to the chip until the field aperture is in focus. It should appear as a dark octagon around the periphery of the image.

- Bring the objective slightly closer, turning the fine focus knob about a quarter-turn. Stop when the thermal grease appears in focus as small, shiny specks.

- Bring the objective closer one last time, turning the fine focus knob exactly one and a quarter turns. Most likely nothing will be in focus. Open the field diaphragm.

- **Scanning for microchannels:**
  - Watching through the eyepiece very carefully, slowly scan left across the chip using the positioning stage joystick. You are looking for a long, blurry feature to pass the screen. This feature is a microchannel. It might be oriented vertically or diagonally. A scan from the chip’s centre all the way left should cross one.

  - If you don’t see one, you will eventually scan to the edge of the chip. When this happens, the image will darken quickly. Scan the chip in the opposite direction to brighten the image once more, and keep looking. This may mean a microchannel passed and you missed it. Try scanning again more slowly. It may also help to close the condenser aperture slightly in order to improve the contrast of the image. If you’re very far up or down from the centre of the chip, the lens may be so far from the nanochannels that you won’t cross the microchannels at all. If you scan in both directions a few times without seeing a microchannel, try re-centering the objective again by looking at the chip’s underside.

  - If you do see one, bring it to the centre of the field of view. Focus on the microchannel using the fine focus knob.

- **Following the microchannel to the nanochannels**
  The nanochannels are at one end of this microchannel. However on first finding a microchannel there is no easy way to tell which.

  - Pick a direction along the microchannel and slowly scan down it. If the image begins to darken, you’re scanning towards a reservoir rather than the nanochannels and you should scan the other way.

  - The nanochannels will appear as a row of much thinner lines running vertically between two microchannels. Centre the field of view on one of the microchannels where it interfaces the array of nanochannels.
- Imaging the nanochannels under fluorescence

With the nanochannels in the field of view you are ready to switch from incandescent to the fluorescent imaging you’ll use to image DNA.

  o Turn on the computer. Open NIS-Elements, the Nikon imaging program.
    Set the temperature of the CCD to -70°C and check the temperature is dropping slowly. Make sure the shutter is closed.

  o Direct the optical path to the CCD (marked L). Turn the mirror on the microscope to the fluorescent lamp (marked B). Turn off the incandescent epi lamp and turn on the X-cite fluorescent lamp, to maximum intensity.

  o Once the CCD has reached -70°C, open the shutter. Set the exposure time to 50s, and the electron multiplying (EM) gain to 999 at 10Hz. Click “live exposure” to watch the field of view in real-time.

  o The microchannel may be faintly visible (but if not, don’t worry). Don’t adjust the positioning stage or focus. Record the X and Y position of the stage in case you move it by accident.

Confining DNA in the nanochannel

The chip is now in place to image DNA. The last step remaining in the setup is to confine a DNA molecule in the nanochannel.

- Turn on the air pump and the pressure meter.

- Gathering new DNA: Open lines 1 and 4, close lines 2 and 3. Open the master line gradually to exert about 100mbar of pressure. This pressure difference will drive fluid from the reservoirs containing DNA across the microchannels to the reservoirs without DNA.

  Within about 2 minutes you should see fluorescing DNA molecules float through the microchannel from left to right. They look like tangled white blobs on the black background.

    o When imaging DNA during setup but not taking denaturation maps, insert the density filter ND4. This will make them fainter but reduce the danger of photonicking.

- Bringing DNA to nanochannels: Open lines 1 and 2, close lines 3 and 4. Open the master line gradually to exert about 500mbar of pressure. This pressure difference will drive fluid between the opposite microchannels, across the nanochannel array.

  Over the course of a few minutes the DNA molecules should slowly migrate across the microchannels towards the nanochannels. (If you don’t see them move in this direction, you’re looking at the microchannel that is a sink for this flow, rather than source. In this case reverse the direction of the pressure gradient: open lines 3 and 4, close lines 1 and 2). Watch the DNA molecules closest to the nanochannels carefully. Eventually they will enter a nanochannel. This is a fast process – the DNA will be sucked into the channel and shoot down it quickly. The
instant you see a DNA molecule enter a nanochannel, close the master line to stop flow through the nanochannels.

- Scan the positioning stage slowly down the nanochannel to find the DNA molecule. It should be stretched into a line. If the molecule isn’t visible, you may have stopped the pressure too late and had the DNA shoot through to the other microchannel. In this case, try again on a new molecule.

Figure 6: A cropped image of a DNA molecule loosely coiled in a microchannel (inset) and stretched in a nanochannel. The white dotted lines show the direction of the nanochannel (although not its width).

Finally, with the microscope fluorescently imaging a single DNA molecule confined in a nanochannel, you are ready to perform a denaturation mapping experiment.

3. Collecting data

Heating DNA to denature it

The confined DNA in its formamide-containing buffer needs to be heated only slightly to denature. While you’re heating it’s a good idea to close the shutter (and open it only periodically to check on the DNA) in order to avoid photonicking.

- Set the power supply to the heating element to a target temperature of 29°C. The thermocouple reading should begin to rise.

- In the meantime, change the capture setting on NIS-Elements to 200 images at 200ms intervals.

- When the temperature reaches 29°C, open the shutter to check the DNA is still in the nanochannel and in the field of view. You may need to adjust the focus slightly. Close the shutter and wait 10 minutes for the DNA to achieve an equilibrium denaturation pattern.
Figure 7: A screen capture of a λ-phage DNA molecule partially denatured in a nanochannel. In contrast to Figure 6, this image shows the entire field of view in order to convey the relative size of the DNA molecule. The denaturation pattern is difficult to discern from one frame. The most obvious feature is a dark stripe in the middle of the molecule. The faint fluorescent line to the right of the DNA is YOYO-1 in a now-empty nanochannel that has unbound from a DNA molecule imaged earlier.

Recording a fluorescence pattern

- Remove the ND4 density filter so that the fluorescent lamp shines with full intensity on the DNA. Open the shutter and focus once more. There should be a barcode of light and dark fluorescence along the DNA molecule. (If not, close the shutter and wait another minute). Capture images.

Collecting data
Keeping the temperature of the heating element at 29C, repeat the procedure of confining new DNA in the nanochannel, waiting for an equilibrium fluorescence pattern, and recording fluorescence patterns. Do this for as many DNA molecules as you can. Eventually the quality of these maps will degrade as the enzymes to prevent photonicking are exhausted (so the DNA breaks into pieces) and the YOYO-1 bleaches (so the DNA becomes more faint). Work until this begins to happen. It should be possible to record fluorescence patterns of 4 or 5 DNA molecules in an hour.
4. Reviewing the data

Checking the denaturation map from a movie

- Using the “nd2 to tif” ImageJ macro, convert the NIS-Elements .nd2 movie files of your denaturation maps to lossless .tifs.

- For each .tif file, use ImageJ to select a rectangle around the DNA molecule. Reslice this region to a stack whose row are the brightest vertical section through the DNA molecule at each frame of the .tif movie. This stack should display the striped pattern of fluorescence on the DNA molecule very clearly.

- From these stacks, pick the one with the longest and most dramatic fluorescence pattern. Save it as a lossless image called stack.tif.

![Figure 8: A stack of denaturation fluorescence patterns of from λ-phage DNA. This stack was made from the same set of images as Figure 7. One row pixels in this stack corresponds to a vertical section through one image (such as Figure 7) taken during a series of image captures.](image)