Last weekend, Greg and I got together to try and combine the chip-coating fluorescent reporter assay I've been developing with the nanoporous tenting he's been working on. The experiment was designed to demonstrate that the volume under a nanoporous tent had been wetted by diffusion of an enzyme (calf intestinal alkaline phosphatase, ciAP) through that volume to the surface of the chip, which had been coated in the ciAP substrate (fluorescein diphosphate, FDP) via poly-L-lysine (PLL). If the enzyme is able to reach the substrate bound to the surface of the chip, it catalyzes a dephosphorylation reaction which results in fluorescent signal from the substrate in the FITC band.

The experimental procedure was outlined beforehand as follows:

Sample Treatments (2x replicates):

Tenting Substrate	Tenting Material
2 um holes	Wafer 1150, 41 nm, 18%
2 um holes	Wafer 4448, 0% porosity
Glass coverglass	none (no tent)
2 um holes	none (no tent)

Assay Reagents:

- Coating solution
  - 10:1 solution of FDP to PLL in DI water
  - 10 uM FDP; 1 uM PLL
  - 100 uL is required for each chip
- Enzyme solution
  - 0.08 ug/mL of ciAP in tris buffered saline (TBS)
  - TBS is 50 mM tris and 150 mM NaCl, pH 7.4
- DI water
  - For rinsing
  - 300 uL required for each chip

Procedure:

Coating

a. Each substrate is submerged face-up in 100 uL of the coating solution in an inverted centrifuge tube cap for between 1 and 1.5 hours.
b. The coating solution is pipetted off and each chip is rinsed 3x with 100 uL of DI water by lifting one end of the tube cap off the bench and pipetting the water with medium speed onto the uppermost corner of the chip so that it flows down the chip surface. The used water is removed between each rinse step.
c. In preparation for the tenting procedure, the chips are placed face-up in a gelbox and slowly exposed to vacuum to dry them.

Tenting

a. Each substrate is placed into gel box
b. Tenting chips are inverted over the substrate, and placed into contact
    i. repositioned so the active area is centered over the active area of the substrate
c. Tents are delaminated using sharp tweezers, pressing against all edges of the tenting chip
    i. Single color, large rectangular sheet is ideal
d. Tenting chips are removed
e. Inspect under microscope

Wetting

a. The substrates are all placed in the same gel box and exposed to room air for a few minutes
b. Gel boxes are closed, and placed in freezer for 15 minutes
c. Inspect gel boxes for cloud of humidity
    i. if no clouding is present, redo the condensation treatment by passing the gel box over boiling water, to capture humidity
d. Minimize time between the wetting and assay steps.

Assay

a. Each chip is again placed face-up into an inverted centrifuge tube cap. The cap is filled with 100 uL of the enzyme solution and allowed to incubate for 5 min.
b. After incubation, the chip is removed from the cap with tweezers and gently pressed face-up onto a small section of silicone sheet stuck onto a glass slide for observation. The slide is then inverted such that the chip surface faces

down and placed into the fluorescence microscope sample holder in a dark environment.

c. The microscope objective is centered and focused on one side of the tented membrane such that half of the image is tented and the other is not (control).
d. Images are taken with the Total Control MATLAB GUI using the FITC excitation lamp, taking care not to expose the samples to the excitation lamp longer than necessary to avoid photobleaching. Fluorescence in the various regions of the images is quantified by imageJ.

Interpretation

a. Each image acts as its own control: the fluorescence observed on the surface adjacent to the tent-covered membrane is the baseline to which the fluorescence of the membrane under the tent is normalized.
b. If the normalized fluorescence under the nanoporous tents is observed to be significantly higher than under the non-porous tents, this is taken as evidence that the space between the tent and the chip has been filled with water and allowed ciAP to diffuse through it.

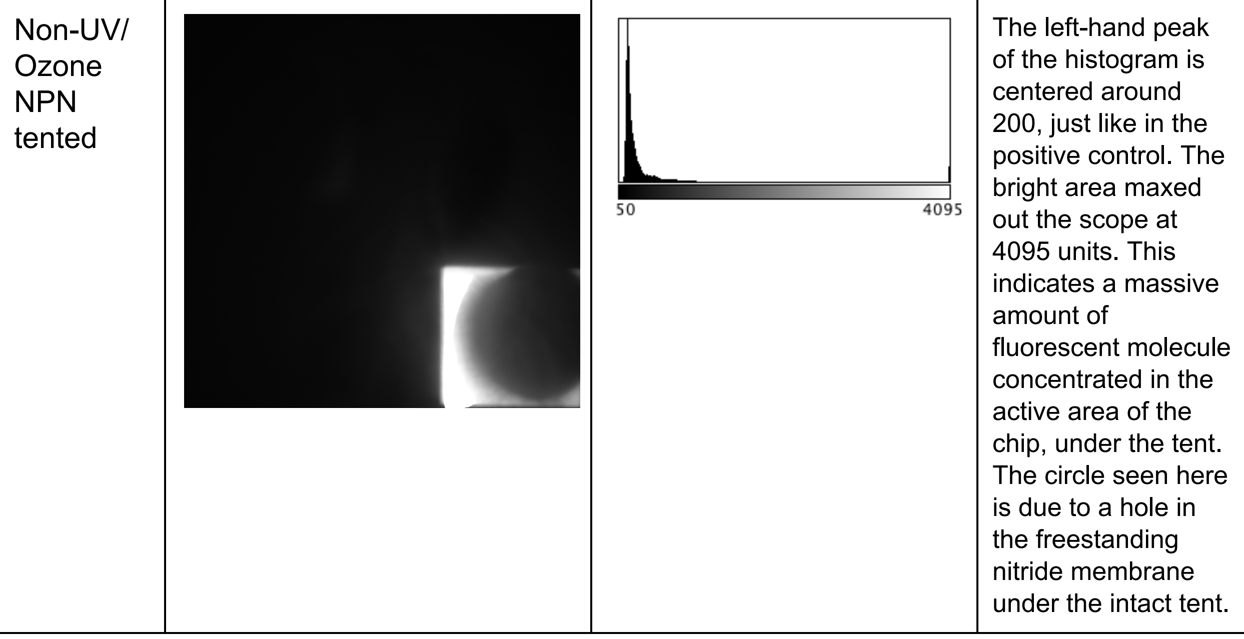
Overall, tenting the membranes proved to be more challenging than what has been shown in the past; many wrinkles formed and some substrates didn't stick very well. The non-porous material in particular had very interesting behavior.

The above image shows a large multi-mm span cut into silicone being bridged by a nonporous nitride dialysis membrane chip (wafer 4448). This material had a Kapton-like veneer when delaminated, without any stickiness. Originally, we thought most thin films should like to stick to a dry substrate and be impossible to remove, but this material did not bond particularly well. It was like a gift ribbon, somewhat stiffer than the NPN that likes to curl on itself.

When we performed this experiment as it was described above on control samples, we were unable to recover any signal from the chip surfaces. To remedy this, we modified the assay procedure: instead of incubating the samples in a centrifuge tube cap containing the enzyme solution, we pipetted 20 uL of the enzyme solution directly onto the surface of the chip, and then gently pressed a glass coverslip overtop of it. This allowed the fluorescent signal to be measured on a wet chip, and resulted in a much healthier signal from control samples.

We stopped the experiment before imaging all the samples due to poor results, but we'll present here the interesting results from the samples we did measure:

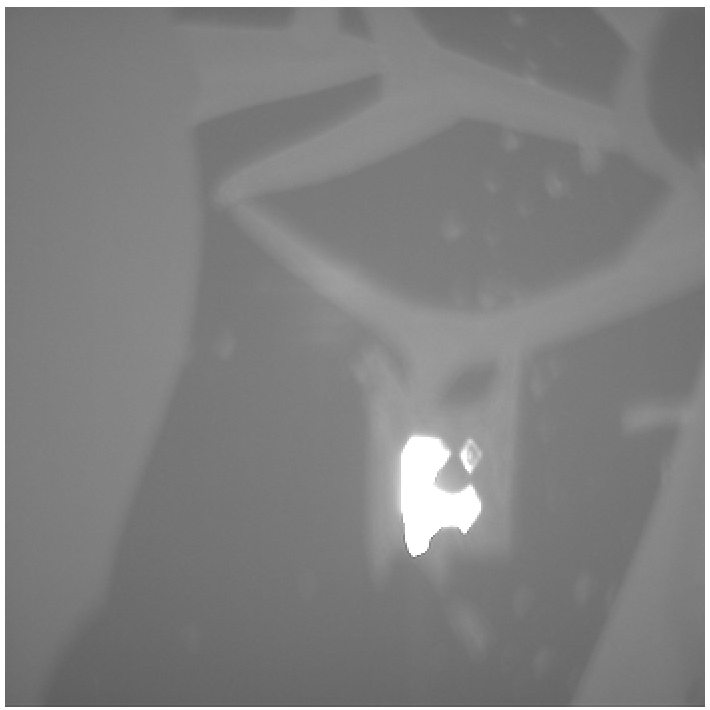
Sample	Representative Image	Histogram	Comments
Untented substrate, dry measured			This surface is indistinguishable from background; very little or no fluorescent signal was captured when measuring on a dried chip.
Untented substrate, wet measured			Positive control. The active area can be made out in the bottom of the image, but the signal is approximately constant everywhere, indicative of enzymatic activity all across the chip surface. This is what we were looking for.



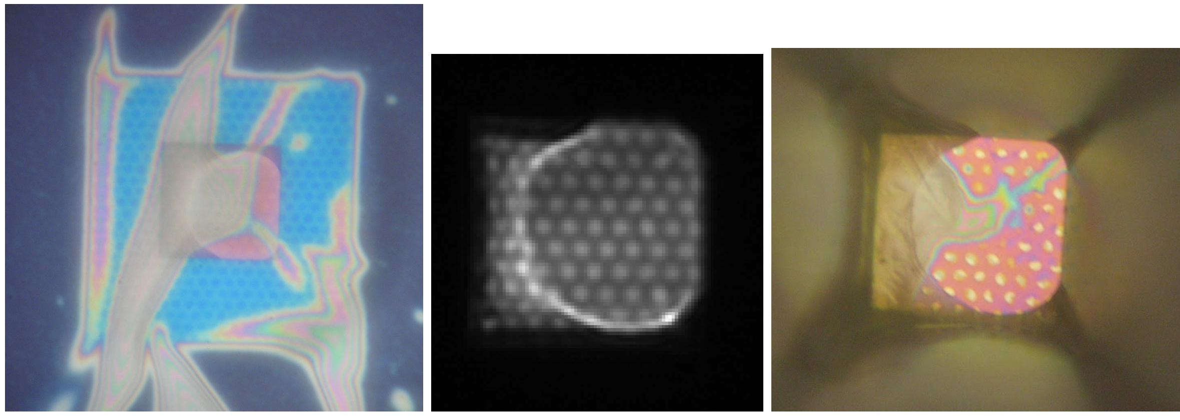
Non-UV/ Ozone NPN tented			The left-hand peak of the histogram is centered around 200, just like in the positive control. The bright area maxed out the scope at 4095 units. This indicates a massive amount of fluorescent molecule concentrated in the active area of the chip, under the tent. The circle seen here is due to a hole in the freestanding nitride membrane under the intact tent.

These images suggest something which we did not anticipate before beginning the experiment: the fluorescein, once dephosphorylated by the activating enzyme, detaches from the poly-L-lysine and becomes soluble, undergoing diffusion. This explains the lack of signal when the chip was treated with enzyme in a 100 uL volume which it was then removed from and checked for fluorescence; the fluorescein diffused out into the bulk solution and remained there when the chip was removed.

In theory, for a successful tenting the maximum fluorescence signal we should expect to obtain from a given area of the tented region of the chip is: (far-field fluorescence * void fraction under tent * transmittance of tent) * volume fraction of tent in the total volume which the measured area is a cross section. A ballpark estimate for the term inside the parentheses is about 30% of far-field fluorescence (measured volume fraction = 34.3%), and it will only decrease when corrected for the volume fraction the tent represents. The bottom line is that we would only expect the signal to decrease from that of the surrounding region.



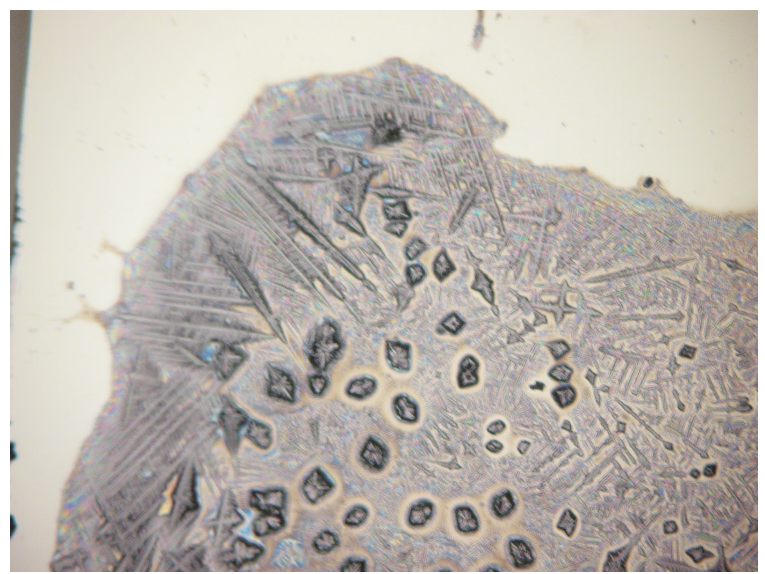
The macroscopic view of the fluorescent porous tent. Wrinkles provide macroscopic fluidic access from the bulk area to the active area, sweeping in fluorophores.



Left: wetted porous nanomembrane, with possible bubble. *Center:* Phase image of the same active area. *Right:* The membrane viewed from the bottom side, through the back well. The 20 nm nitride film appears torn and wrinkled, while the 200 nm oxide layer appears intact. The oxide layer may have become detached from the nitride film in the wrinkled region, allowing water or air to wick out of the nanocavities.

Insert Evaporative video here

The fluorescence in the previous images appears to have been localized to the wrinkled region. As the membrane is dried out, water retreats from the wrinkled region. We believe the increase in fluorescence is due to water evaporating near the torn membrane and leaving behind mobile fluorophore



Dried out substrates leave a crystalline residue. We think it is the protein residue, and not ice crystals.

What did we learn?

- Fluorescent product is diffusive
- Not all tenting materials are created equal.
    - There are probably some excellent mechanics studies that we can do that will allow us to determine whether a material will lay flat or not.
    - Some nanoporous nitrides wrinkle, others do not. The non porous material was particularly non-stick, but also strong enough to bridge a 3 mm wide gap without tearing.
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What might we do in the future to improve the fidelity of this experiment?

- Swap the reactive products of this experiment around, coating the substrate with ciAP and the letting the FDP diffuse
    - This should increase the signal strength of the reaction

- Use a substrate with posts
    - This should increase the signal from the substrate by virtue of having more surface area underneath a tent (comparing favorably with the wrinkles we have previously observed).

- We need a better way to control the delamination; tweezers have just too much error.
    - A carefully designed metal blunt spudger should do the trick if placed on a mechanical lever.