Scanning the Controls: Genomics and Nanotechnology

Robert H. Austin, Jonas O. Tegenfeldt, Han Cao, Stephen Y. Chou, and Edward C. Cox

***Abstract*—We outline some of the possible applications of nanotechnology to modern molecular biology and discuss several technologies that can be used to make nanoscale confining environments (channels or post arrays) for long polymers such as DNA. A particular emphasis is placed on making large arrays using non-electron beam lithography methods. We then discuss how focused ion beam (FIB) milling can be used to construct nearfield slits for examining molecules.**

***Index Terms*—DNA, focused ion beam (FIB), nanobiology, nanofabrication, nanoimprinting.**

I. Introduction

THE integrated circuit revolution, made possible by microfabrication technology, is just now entering the world of biology. Some of the authors are old enough to have built a Heath Kit tube tuner and amplifier and to notice that the transfer characteristics of a field-effect transistor (FET) resembles a triode. So, we know the revolution that can happen. The same kind of revolution that occurred in electronics may in fact occur in biology. The world of biology is inherently on the micron and below scale and this is where micro/nanofabrication lives. We can process, examine, and move biological objects at their natural length scale. Furthermore, a great deal of the complexity of biology comes from heterogeneity: no two objects are alike. Sometimes, the rarest one is the most interesting. Microfabricated devices, which basically are "flatlanders" can find those rare ones, be it a single molecule or a single cell.

Although there are many possible applications of nanotechnology to biology, in this article we would like to focus on one subject, the arrangement of control proteins on DNA within a cell. As we will discuss, it is these controls that make a particular cell what it is and determine the past and predict the future of the cell. Although we have all seen pretty pictures of the DNA polymer as a double helix, the actual configuration on DNA within a eukaryotic cell is much more complex than this cartoon picture. We have about a meter of DNA packed into a five micron diameter nucleus and the complex entanglement of such a huge length of DNA in a small volume is a stupendous problem when it comes to reproducing that strand. The packaging of the DNA within the chromosome is by no means a trivial task: those of you that like to fish can appreciate the potential problems. On top of simple entanglement are the beautiful problems arising from the topology of strand reproduction, since DNA as a double helix is topologically linked about every 10 basepairs. You cannot remove links without cutting the DNA and reselaing it. Here, specialized proteins play a critical role in binding to the DNA and untangling this Gordian knot [1].

On top of the mechanical and topological complexity is the complexity of the genome itself, consisting of about one billion basepairs. The Human Genome Project and Celera Genomics Corporation have both completed an initial sequencing of the human genome—the genetic blueprint for human beings. Former President Clinton congratulated the scientists working in both the public and private sectors on this landmark achievement, which promises to lead to a new era of molecular medicine, an era that will bring new ways to prevent, diagnose, treat, and cure disease.

This enormous project was done about three times faster than the most optimistic estimates in the early 1990s and for about one tenth of the estimated price. Basically, brute force robotics and computer developments did it, along with some knowledge of physics. However, we are far from understanding how this enormous collection of bases translates into a working cell. An excellent statement of this fact can be found in the recent Stanley Fields article in Science [2]:

> If the architect you hired to design your home brought you a blueprint that solely consisted of a long list of parts that began "windowwabeborogovestaircasedoorjubjub…," you might start to wonder if and when you will see your new house. Some people have similar reservations about the recently "completed" human genome sequence, heralded as the "genetic blueprint" that will revolutionize biology and medicine. Deciphering how a mere 10^7 nucleotides result in a yeast cell—let alone how 3×10^9 nucleotides result in Tiger Woods or Britney Spears—cannot begin until the genes have been annotated. This step includes figuring out the proteins that these genes encode and what they do for a living. But understanding how all of these proteins collaborate to

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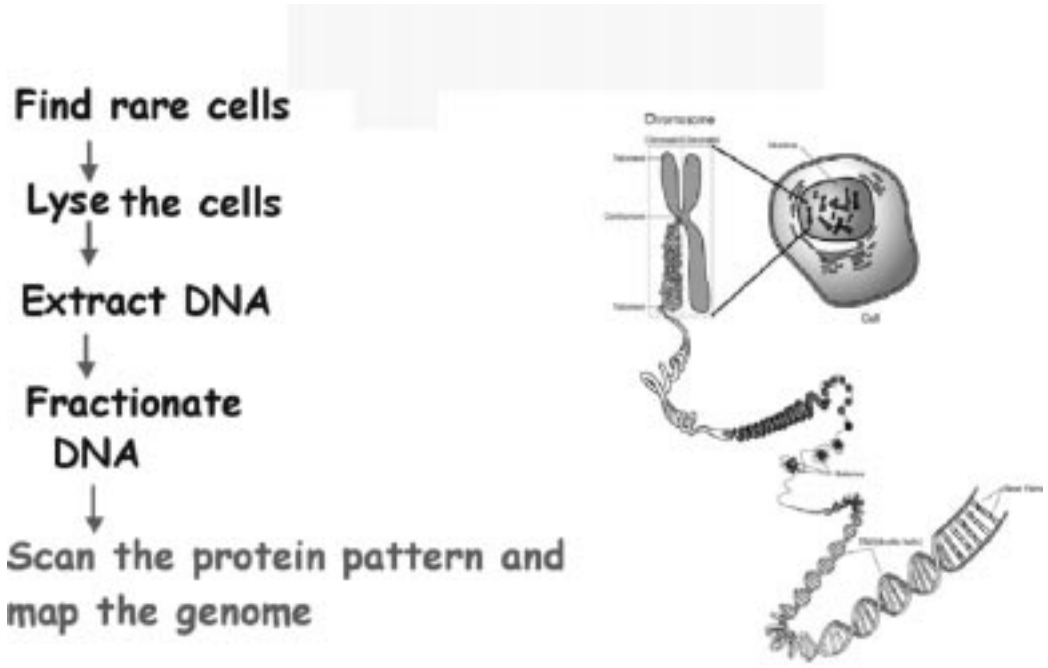

Fig. 1. A cartoon of what we are attempting to do: move from a cell down to the sequence of DNA in the cell.

carry out cellular processes is the real enterprise at hand. An interdisciplinary spirit will come to guide those excited by the global analysis of protein function. Geneticists need to talk to chemists, physiologists to physicists, cell biologists to computer scientists. With questions so grand, the expertise to answer them requires the entire spectrum of science. This combination of new technology and its widespread dispersion together with broad-ranging collaborative projects will culminate in the "frabjous day" when the undertaking that began with genome sequencing reaches fruition.

That is, although the human genome has now been "sequenced," at some level, it is misleading to think that we now understand how the genome is expressed and how the cell functions. The key word here is "epigenetics" [3]. Epigenetics refers to the nongenomic information that a cell passes on to its progeny. This "information" can vary from methylation of the DNA bases to the occupancy of the promoter and repressor sites on the DNA that control gene expression (see the 293 volume of Science, 10 August for a current look at these issues). In our lab, we are trying to do it all: select rare cells, extract the genomic material, fractionate the genomic DNA, and scan the DNA for the occupancy of the epigentic control sites. The Big Picture we are pursuing in our labs is cartooned in Fig. 1. It is a challenging task.

II. Scanning the Controls

Modern molecular biology has provided us with some wonderful tools which allow us to make very specific probes of biological function. One of the most marvelous is the "green fluorescent protein" (GFP), a single stranded amino acid polymer which when properly folded and upon completion of internal chemical bonds [4] becomes highly fluorescent. This fluorescent protein can then be used as a tag to indicate the expression of a gene. In our case, we have created a "fusion" protein where the code for a GFP protein is added to code for a control protein, so that when the control protein is expressed and binds to a regulatory site on DNA that position is marked by the fluorescent GFP part of the "fusion protein." The epigenetic part of our work using nanofabrication is the scanning of the genomic DNA for the pattern of occupied control sites using the technology of these fusion proteins between GFP and selected control proteins. Our model system is the lac operon, which controls the lactose metabolism of *E. coli*. The lac repressor (lacI) is a transcription factor that binds to the DNA and prevents the transcription of the operon [5]. Our mission is to see if we can find the pattern of occupied sites on the DNA and the position of these occupied sites to as high a resolution as possible. This varies from the size of a typical promoter site, about 10 nm, to the size of a typical gene, about 100 nm.

The challenge is to excite the molecule locally at a length scale as small as possible. In free field, this difficult optically at a length scale smaller than the wavelength, λ. We have pursued near-field techniques. The basic idea is to BACK illuminate a slit nanofabricated in a thin aluminum film and excite fluorescent tags as a stretched DNA molecule passes transverse to the slit in the evanescent field [7]. In this way, the resolution of the lac repressor molecules bound to the DNA can be substantially improved over the normal limit of the wavelength of the light. Although the actual problem of determining the near and far field components of the evanescent wave propagating through a slit much smaller than the wavelength of light is a difficult problem, as a rule of thumb the resolution is given roughly by the slit width if the light is polarized parallel to the slit and the far-field transmitted light falls off exponentially as $\exp[-w/\lambda]$ for a slit

of width w. This part of the physics is pretty straightforward, but the biological molecules pose more difficult problems.

In our case, the problem is that the persistence length p of dsDNA is relatively quite small, about 50 nm, or about 150 basepairs. This means that a length $L \gg p$ of dsDNA actually looks like a ball whose radius of gyration $R_g = \sqrt{Lp/6}$ [6] is far smaller than the length: a 16-μm long λ phage DNA molecule forms a ball whose diameter is only 0.5 μm. The task is to straighten out this ball and then keep the molecule straightened out when entropy wants to curl it up. You may remember from a polymer statistics that the basic shape is that of a flower: the head is dis-ordered, followed by elongation [8]. There are, thus, two parts to the high resolution imaging of single DNA molecules: we need to have both *nanoslits* for high spatial resolution and *nanochannels* to confine the DNA and keep it straight. Although our original publication using nanoslits indicated that there near-field excitation might work, it also highlighted two main problem areas.

1) Poor stretching of DNA. The ends are clearly disordered in the DNA since the DNA is moving in channels much larger than the persistence length of the DNA. By forcing the DNA into channels that are on the order of the persistence length (50 nm), the DNA is forced into a uniformly stretched confirmation
2) Poor optical resolution (200 nm). The nearfield intensity and resolution falls off rapidly with distance from the nearfield slits. Smaller channels force the DNA to close proximity of the slits increasing the intensity and the resolution of the nearfield excitation.

Thus, although the first pictures of the fabricated nanoslits were pretty and we did succeed in getting very preliminary results, there is a great deal of work to be done now that the first steps have been taken. The following list is of the three main projects that we are attacking to address and we will spend the rest of this paper discussing these three subjects:

1) Nanoimprinted arrays;
2) Nanofabrication of channels as well as slits, using focused ion beam (FIB) technology and nanoimprinting;
3) "Unzipping" polymer technology to create buried internal channels.

A. Nanoimprinted Arrays

One of the early lessons you learn when you cross over into real biological systems is that rarely are two biological objects identical, they are quite often different. Any attempt to find patterns within a biological system, such as in our case scanning the genome for the proteins that bind to the promoter sites, must be designed to look at a large number of supposedly identical molecules which may have a large variance. We have been aware of this from the start and our original design used photolithography to make arrays of channels. However, photolithography cannot be used to make submicron width channels easily and electron-beam lithography is tedious and an expensive way to make nanochannels over large (cm $\times$ cm) areas. One of the authors of this article, Chou, has developed nanoimprinting lithography (NIL) to transfer arrays of nanochannels into substrates over large areas. Cao, from the Chou group, has developed ways to narrow the channels to well under 100 nm.

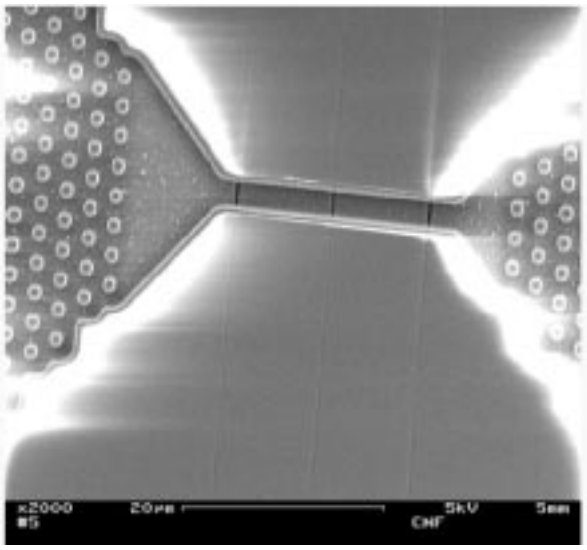

Fig. 2. Scanning electron microscope image of a 100-nm-wide slit nanofabricated into a etched quartz structure, with a 5-μm-wide channel for DNA passage. The array of posts at the end were designed to straighten the DNA molecules.

Chou's technology has been well documented in the literature [10] and [11] and we will not repeat the details here. The fundamental way that the channels are initially created is by creating an interference pattern using two beams from an argon laser ($\lambda = 351$ nm) on a photoresist. With careful adjustment of the parameters this can create channels which are 100 nm across. Fig. 3 shows an array of channels that can be constructed in this manner. However, these channels are still too wide to fully stretch a DNA molecule.

There are however ways to narrow these channels. More details will be published in a paper in preparation [9], but we can sketch one promising technique here. In order to make ultrasmall nanofluidic channels suitable for single biomolecule analysis, we have tested a number of deposition processing methods to further reduce the trench width of the channels fabricated by NIL. Since the depth of channels could be always reduced by decreasing etching rate and time, the main concern is to narrow the channels. Fig. 4 shows a nanochannel grating with original trench width of 100 nm narrowed down to 50 nm by controlled sputtering of SiO_2 or other material at various angles. Other materials can also be used. Channel dimensions and geometry can be controlled by varying the process parameters and the technology is easily scalable to large arrays over entire wafers.

B. Nanofabrication of Slits Using FIB Nanomachining

Fabrication of nanoslits can be done in several ways, we have explored two of them. The first way is the traditional way of ebeam lithography of ebeam resists followed by development and reactive ion etching. This is a time-proven technology, but has the disadvantage of being time consuming, difficult and can be expensive. However, it does work and we have succeeded in making true nanoslits. A picture of these nanoslits was shown in Fig. 2. We have been able to characterize the transmission of

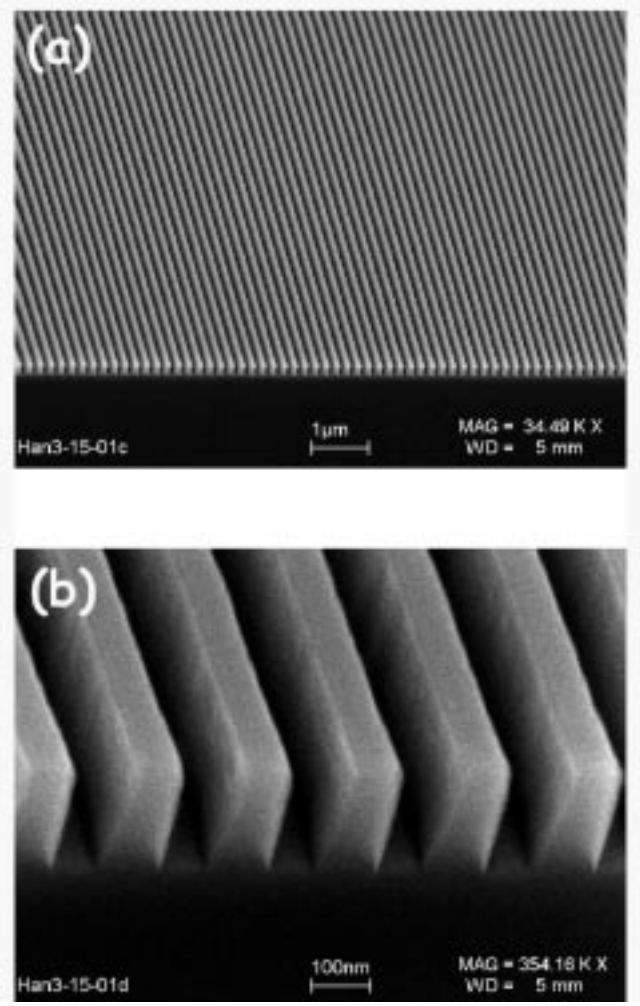

Fig. 3. Array of channels etched into quartz. Parts (a) and (b) are at two different magnifications. The channel pattern was imprinted into a polymer which acted as a reactive ion etching (RIE) etch mask.

these nanoslits as a function of polarization and wavelength, so many aspects of these slits are well characterized if not understood at present.

The other way to make nanoslits is by direct nanomachining using a FIB tool. A FIB uses a beam of energetic ions such as Ga+ to sputter material away and is capable of resolution down to 20 nm and can etch down many microns in principle. The huge advantage to FIB is that it is basically "what you see is what you get:" immediately after FIB milling the structure you can image the nanofabricated slit and see what you have. There are some problems with this technique, such as implanting of the Ga+ ions in the substrate which can reduce the optical transmission of the substrate, but the problems seem to have solutions. Recently we have good success using enhanced etching with gas mixtures which chemically enhance the etching rates. This is a two-step process: aluminum is first etched using the normal (no gas) sputtering ion beam, then a wider area is etched using a gas specifically designed to etch oxides. The resulting etch rate of the oxide is a factor of 10–50 greater than the etch rate of aluminum, so that by careful timing of the etch times we can remove the gallium doped quartz under the aluminum film without

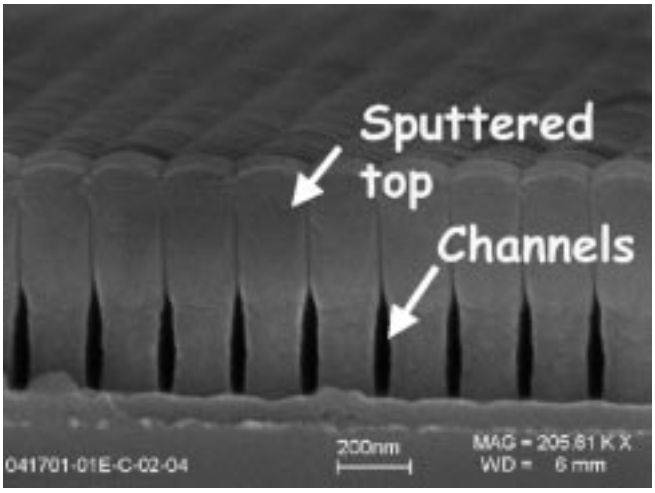

Fig. 4. Arrays of posts fabricated by Nanoimprinting Lithography and sealed by sputtering (work done by Zhaoning Yu, Nanostructure Group).

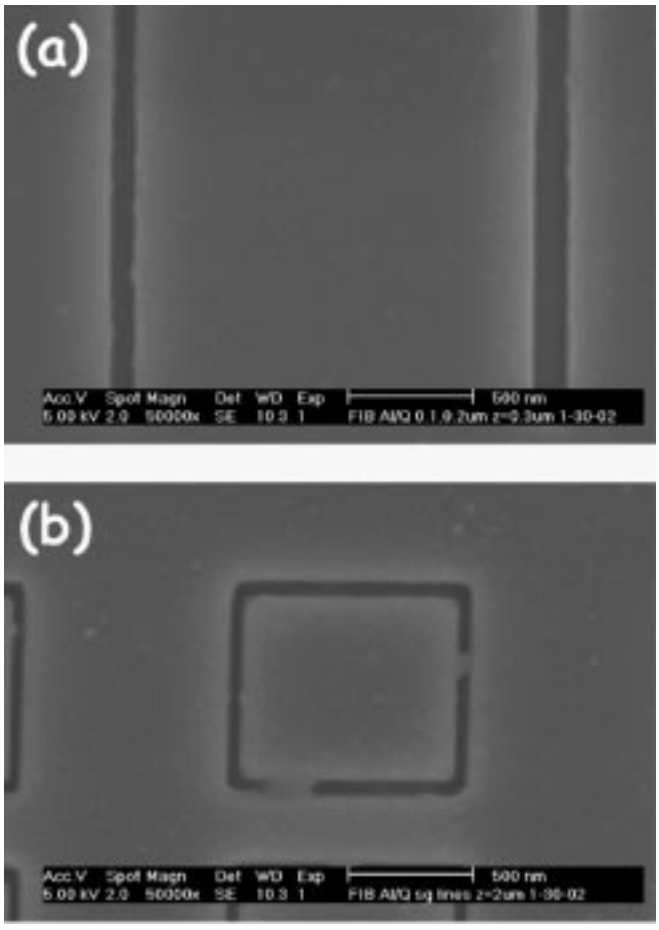

Fig. 5. FIB-milled nanoslits in a 50-nm thick aluminum film. (a) 100-nm straight lines. (b) 50-nm-wide lines drawn as a square. At a few points the aluminum is not milled through and a metal neck can be seen.

substantially increasing the width of the aluminum lines themselves. It has also proven possible by careful timing of the ion milling beam to remove the aluminum without implantation of Ga in the underlying oxide.

Fig. 5 shows some recent FIB milled slits that we have fabricated in aluminum on quartz and Fig. 6 shows preliminary tests

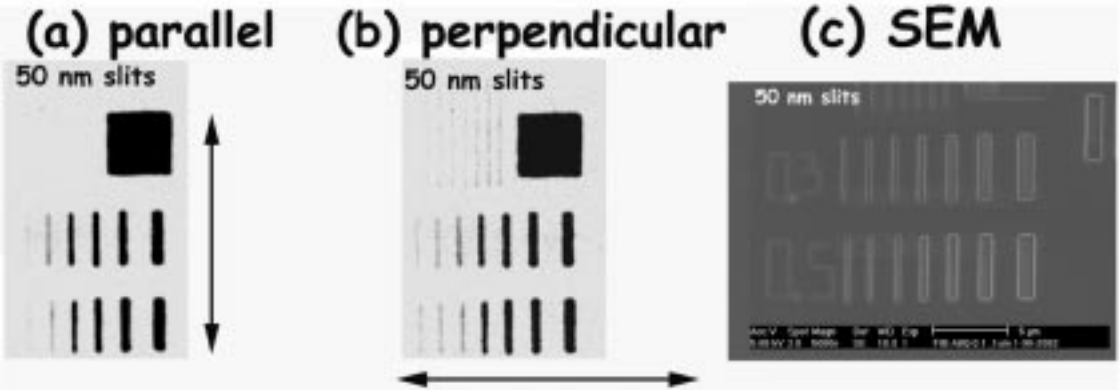

Fig. 6. (a) and (b) Far-field optical transmission as a function of the electric field polarization through FIB milled slits ranging from 1-μm width through 50-nm width. The contrast has been inverted for ease of visualization. (c) Scanning electron microscope (SEM) image of the FIB milled slits.

of the optical transparency indicate that we have good transmission. In Fig. 6 plane polarized light illuminated the underside of a wafer and the amount of transmitted light was observed on the top side. As the plane of polarization of the light is rotated, the amount of far-field light that can penetrate the slits changes. If the light is polarized perpendicular to the slits the electric field can induce a large dipole moment across the slits and far-field light can be observed, while if the light is polarized parallel to the slits there is no induced electric dipole and no far-field emission.

C. "Unzipping" Polymers for Self-Sealed Channels

"Unzipping" polymers are polymers which undergo a phase change directly from solid to gas at some well characterized temperature. A layer of such a polymer can be nanomachined to form structures, chemical vapor deposition techniques can be used to cover the nanostructures with quartz (for example), then the polymer can be unzipped, leaving behind sealed nanostructures. Professor Paul Kohl of Georgia Tech University has pioneered this technology [12] and [13] and in collaboration with B.F. Goodrich we have worked with our graduate student Li to make micron-sized structures and with Chou's group we have been able to use the imprinting techniques to transfer nanoscale structures in the unzipping polymer.

At present we have worked with polynorbornene (PNB), a polymer with a rather high unzipping temperature of 430 °C. We have successfully made structures, it is a difficult process requiring ultraclean controlled atmosphere with less than 1 ppm O_2 and careful temperature control both in the ramp-up times and the precision of the temperatures obtained, accuracy to 1 °C and stability to 1°C is extremely useful. We have used a special formulation of polynorbornene developed by B.F. Goodrich called Unity and followed the protocol developed by Professor Kohl. We have basically developed PNB nanostructures in two ways, first using more conventional ebeam lithography and then using the imprinting technology of Chou's group.

We present two images of PNB structures that have been constructed on the nanometer scale. The first structure, Fig. 7 was made a by a combination of conventional optical lithography and the isotropic aspect of reactive ion etching (RIE) on small structures. 50-μm-wide structures were connected via

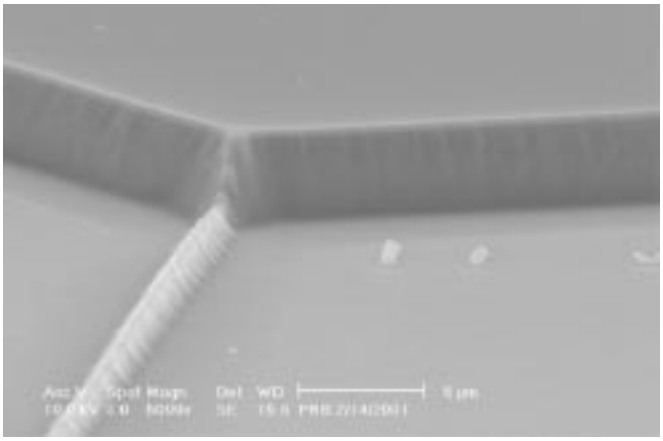

Fig. 7. SEM view of polynorbornene channels covered with SiO_2.

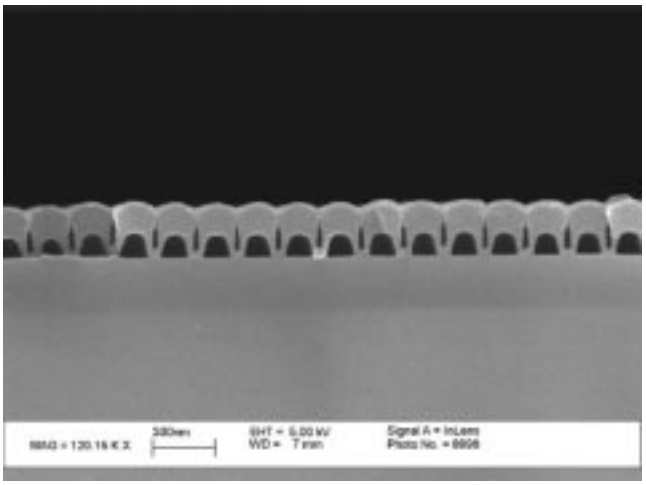

Fig. 8. SEM view of the edge of a wafer. The surface has open channels which where originally polynorbornene filled covered with SiO_2 before unzipping.

1-μm-wide channels, but the isotropic RIE etching on the 1-μm-wide channels resulted in a final diameter of 100 nm. The next structure, shown in Fig. 8 is 100-nm-wide channels made by imprinting a pattern of lines into a polymer film spun over polynorbornene and reactive ion etching the lines down. In both cases plasma-enhanced chemical vapor deposition techniques where used to cover the polynorbornene with SiO_2. Fig. 9 shows

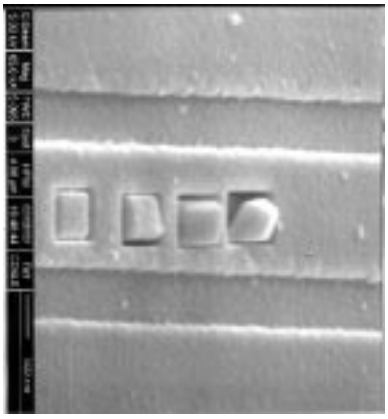

Fig. 9. SEM view of open channels created using polynorbornene unzipping which have squares cut into them using a FIB. The deepest cuts at the top have had the free squares fall into the channel.

the roof of a 1-μm-wide PNB channel which has been coated with aluminum and 100 nm of SiO_2 and then squares have been successively cut into the top of the channels using FIB nanomachining. As you can see in Fig. 9, at some cut thickness the roof of the channel is pierced and the now free piece falls into the hollow channel, either due to gravity, stress forces between the SiO_2 and the silicon substrate used here or the Casimir force due to vacuum fluctuations of the electromagnetic field [14].

III. Conclusion

We have tried to outline in this paper the reasons why nanofabrication has a clear future in modern molecular biology, but that there are strong technological challenges to be addressed. We have confined ourselves to a subset of these technological problems and have discussed how nanotechnology can be used to to align, straighten and examine the proteins binding to DNA at ultrahigh spatial resolution.

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Dr. Chou has received a number of awards for his research and has served as a committee member on numerous international conferences.

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