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Point of VIEW

Sensing Single Base Incorporation with Nanopore Micromanipulation

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ABSTRACT Using single-molecule techniques, scientists can routinely investigate the action of a single enzyme. The goal of such studies is usually to gain accurate information unachievable in ensemble assays, such as the maximum instantaneous rate of reaction, the existence of pauses or backward steps, *etc*. In the article discussed here, the authors have increased their experimental sensitivity so that they can detect a single enzymatic cycle. This improvement makes possible the use of a polymerase enzyme to sequence a single DNA molecule.

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During DNA replication, a DNA polymerase couples with an astonishing fidelity each base on one strand with its Watson–Crick complement, which it then ligates to the growing complementary strand. If one could follow this tremendous nanomachine and determine the identity of each incorporated nucleotide, an extremely efficient sequencing tool would be constructed that did not require sample amplification and the biases it may generate. Extremely ingenious experimental setups have been used in many single-molecule experiments to attempt this goal, but very few have gone beyond a proof-of-principle and shown practical feasibility. One approach relies on the detection of the fluorescence of the last nucleotide incorporated by the polymerase, as proposed by Webb *et al*. (*1*) and Quake *et al*. (*2*). A second micromanipulation approach is based upon measuring the mechanical displacement produced by the nucleotide incorporation. Indeed, two major difficulties have to be overcome in this approach: one needs to track the position of a single polymerase with a resolution better than the size of a single base (3.4 Å) and to identify the type of the incorporated base. In a recent study, researchers at the Scripps Oceanographic Institute report on a major step toward this goal. Their system involves a small current flowing in a nanopore inserted in a phospholipid bilayer. The progression of DNA replication is monitored as the current is perturbed by a DNA molecule threaded through the pore.

In this article, Cockroft *et al*. (*3*) present a clever molecular design based on α-hemolysin that solves the two main difficulties mentioned above. α-Hemolysin is an enzyme used by *Staphylococcus aureus* to punch tiny holes in the phospholipid membrane of its targets. This enzyme has the shape of a mushroom with a 1.5 nm diameter central channel. It is possible to isolate this nanopore and incorporate it into a phospholipid membrane that separates two vessels, each equipped with an electrode. In the absence of α-hemolysin, the membrane is a perfect insulator, and no current flows when a voltage is applied to the electrodes. A single α-hemolysin integrating into the membrane is easily detected by the current surge through the membrane. Ten years ago, researchers had proposed using this device to sequence DNA (*4*): the idea is based on the fact that a single-stranded DNA (ssDNA) is slim enough to thread through the pore and to significantly reduce the current. The hope was that changes in the DNA sequence would alter the current intensity. Unfortunately, two phenomena degrade the signal and prevent the determination of the sequence. First, the length of the nanopore accommodates several bases at the same time, making the current strongly averaged across the sequence. Second, ssDNA propelled by the electric field passes too rapidly (10^5 nt/s) through the nanopore. So far, sequence-dependent current modulations have been measured using homopolymer or repeated DNA sequences (polyA-polyC (*5*)). With

natural (or random) polynucleotides, the signal-to-noise ratio was by far too small to provide relevant information on the sequence. In their paper, Cockroft *et al.* still monitored the current modulation produced by an ssDNA moving in an α-hemolysin pore, but to improve the signal-to-noise ratio, they used two tricks. First, they blocked the molecule by providing a cap at both ends so that the ssDNA could not escape after being read through the nanopore. Second, they engineered a hybrid polymer of ssDNA on one end linked to a short poly(ethylene glycol) (PEG) on the other end. It turned out that the current flowing through the nanopore varied significantly on whether the molecule in the channel was ssDNA or PEG (presumably because they differ in size). This nanodevice displayed its maximum sensitivity when the junction between the two polymers was in the middle of the pore channel. In that situation, the authors could monitor a shift of the molecule corresponding to 2.4 Å, enough to determine whether a polymerase had incorporated a single base.

As seen in Figure 1, the caps used in this experiment were of course molecular: at its 5′ end, the ssDNA was coupled to a biotin moiety *via* a short PEG linker; by binding streptavidin to the biotin, the authors ensured that the ssDNA could only enter the channel *via* its free 3′ end. Once threaded through the channel, a short complementary ssDNA oligonucleotide was hybridized to the 3′ end of the DNA to be sequenced. The resulting double-stranded DNA (dsDNA) was too large to pass through the nanopore, resulting in an ssDNA molecule that was forced to span the channel. It could move back and forth until it was blocked either by the streptavidin or by the dsDNA. The authors could force the molecule into either of these two states by applying a positive or negative voltage across the membrane. The molecule lengths were chosen so that when the molecule was blocked by the dsDNA, the ssDNA–PEG junction was

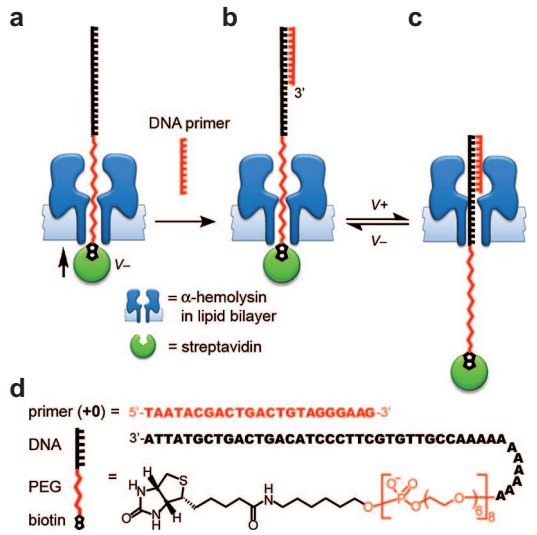

Figure 1. Principle of the experiment. An ssDNA is coupled to a biotin moiety *via* a short PEG linker. a) By binding streptavidin to the biotin, the authors ensure that the ssDNA can only enter the channel *via* its free 3′ end. b) Once threaded through the channel, a short complementary ssDNA oligonucleotide is hybridized to the 3′ end of the DNA. c) The resulting dsDNA is too large to pass through the nanopore, resulting in an ssDNA molecule, which is forced to span the channel. Moreover, when the ssDNA–dsDNA junction is positioned in the center of the channel, the authors can monitor a shift of the molecule corresponding to 2.4 Å, enough to distinguish that a polymerase has incorporated a single base. (Reproduced with permission from *J. Am. Chem Soc.* 2008, *130*, 818–820. Copyright 2008 American Chemical Society.)

right at the center of the channel, providing maximum detection sensitivity. The authors refer to this as the monitoring position. When the polarization was switched to a negative value, the molecule slid back and was blocked by streptavidin, and the junction between ssDNA and dsDNA became accessible to a DNA-polymerase. This situation is referred to as the elongation position, because the polymerase could then add one or more bases to the dsDNA. To detect such an event, the authors switched back to the monitoring position and compared the current flowing through the obstructed channel to the value measured before the polymerase action. Because a polymerase will only incorporate nucleotides that are provided in the solution, the authors knew the identity of the sequence. By switching back and forth from the elongation to the monitoring position while changing the nucleotides in solution, the authors recorded the addition of bases one by one.

This experiment provides an alternative to the impressive experiment performed in 2006 in Steve Block's laboratory at Stanford (*6*). In their study, Block *et al.* pushed the spatial resolution of their optical tweezers to the ultimate angstrom limit in order to detect the pausing of an RNA polymerase each time it encountered a base for which the concentration of the complementary nucleotide had been depleted. By repeating this experiment several times while changing the nucleotide at low concentration, the Stanford group was able to sequence a single DNA molecule. In both experiments, the number of bases sequenced is very small (10 in the current work, 30 in the Block assay) and definitely not competitive with traditional biochemical sequencing or pyrophosphorolysis approaches. Nevertheless, these two experiments have succeeded in achieving a major conceptual breakthrough: the measurement of polymerase activity with single-base resolution. In both experiments, increasing the size of the sequenced DNA will not be easy. This is especially true in this new study because the nanopore sensitivity requires the presence in the channel of a hybrid (DNA–PEG) molecule of varying proportion. The length of sequenced DNA is thus proportional to the length of the channel pore. The present sequencing time scale also

needs to be improved, but these amazing experiments have already provided very interesting tools for studying the mechanism of polymerases.

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