The NTD Nanoscope

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Abstract

Nanopore transduction detection (NTD) offers prospects for a number of highly sensitive and discriminative applications, including: (i) single nucleotide polymorphism (SNP) detection; (ii) targeted DNA re-seqencing; protein isoform assaying, and biosensing via antibody or aptamer coupled molecules, as will be discussed.

The NTD 'Nanoscope' functionalizes a single nanopore with a channel current modulator that is designed to transduce events, such as binding to a specific target. Nanopore event transduction involves single-molecule biophysics, engineered information flows, and nanopore cheminformatics. Current SNP assaying, SNP discovery, DNA sequencing and RNA-seq methods are typically limited by the accuracy of the error rate of the enzymes involved, such as methods involving the polymerase chain reaction (PCR) enzyme. The NTD Nanoscope offers a means to obtain higher accuracy as it is a single-molecule method that does not inherently involve use of enzymes, using a functionalized nanopore instead.

The NTD Nanoscope offers the means to: (1) complete the SNP and DNA and RNA-seq sequencing pipeline with error rate rarer than one-in-a-thousand; (2) offer a variety of glycoform and isoform discerning methods, perhaps as a postprocessing step on Gel bands, to 'split' the bands into their sub-components; and (3) a variety of conformational state discerning methods, for critical information encoded in protein conformational freedoms and kinetics antibody effector activation, for example, or disease conformations, such as for prions, for example.

Introduction

The NTD Nanoscope offers the means to critically complete the SNP and RNA-seq data processing pipeline. Current methods used by industrial for DNA seqeucing have error rates of approximately one-in-a-thousand. This is a limitation due to the enzymes used in the methods themselves having error rates of approximately one-in-a-thousand. DNA sequencing is fast becoming incredibly inexpensive, but at this one-in-a-thousand error rate. The problem is that disease conditions more rare than one-in-a thousand can be overlooked or mis-diagnosed, and most genetic diseases are more rare than this. What is needed is targeted re-sequencing and SNP discovery in the 'important' parts of the genome to obtain reduced error rates, and to do so in an inexpensive way.

In the NTD functionalization the transducer molecule is drawn into the channel by an applied potential but is too big to translocate, instead becoming stuck in a bistable capture such that it modulates the channel's ion-flow with stationary statistics in a distinctive way. If the channel modulator is bifunctional in that one end is meant to be captured and modulate while the other end is linked to an aptamer or antibody or ssDNA for specific binding (or annealing), then we have the basis for a remarkably sensitive and specific event transducer.

The NTD Nanoscope offers a means to do sensing with better than one-in-a-thousand accuracy on any SNP variant or antigen. The NTD Nanoscope description in what follows also directly establishes a general-target (non-DNA) biosensor with sensitivity and specificity limited by that of the aptamer or antibody involved.

A nanopore can be used for detection purposes by using translocation /dwell-time (T/DT) based approaches, which often rely on blockade dwell-times; and the nanopore transduction detection (NTD) based approaches, which functionalize the nanopore by utilizing an engineered blockade molecule with blockade features typically *not* including dwell-time (or translocation). The NTD approach offers prospects for highly sensitive and discriminative biosensing by functionalizing a single nanopore with a non-translocating channel current modulator that is designed to transduce events, such as binding to a specific target.

Translocation/dwell-time methods introduce different states to the channel via use of the frequency of channel blockades, from a series of individual molecular blockades (often during their translocation). A common feature employed in translocation/dwell-time discrimination, and often the only feature, is the blockade dwell-time where the dwell-time is typically engineered to be associated with the lifetime until a specific bond failure occurs. Other dwell-time feature variations include time *until* a bond-formation occurs, or simply measuring the approximate length of a polymer according to its translocation 'dwell'-time. For translocation-based approaches, blockade 'level' is usually a fixed level, and often not stationary,especially if one is trying to elicit non-stationary sequence information from the stationary blockade – whereas NTD tracks the non-stationary sequence information via corresponding phases of stationary statistics.

Transduction methods introduce different states to the channel via observations of changes in blockade statistics on a single molecular blockade event that is modulatory. This is an arrangement involving a partially-captured, single-molecule, channel modulator, typically with a

binding moiety for a specific target of interest linked to the modulator's extra-channel portion. The modulator's 'state' changes according to whether its binding moiety is bound or unbound.

The nanopore transduction detection platform (Fig. 1) involves functionalizing a nanopore detector platform in a new way that is cognizant of signal processing and machine learning capabilities and advantages, such that a highly sensitive biosensing capability is achieved [83]. The core idea in the NTD functionalization of the nanopore detector is to design a molecule that can be drawn into the channel (by an applied potential) but will be too big to translocate, instead becoming stuck in a bistable 'capture' such that it modulates the ion-flow in a distinctive way (Fig. 4 shows some controls). An approximately two-state 'telegraph signal' has been engineered for a number of NTD modulators.

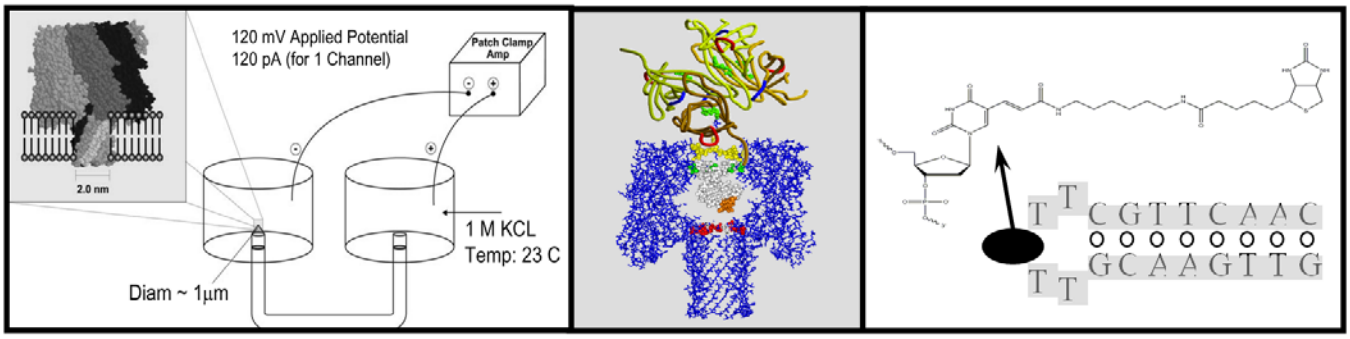

Figure 1. Schematic diagram of the nanopore transduction detector. The nanopore detector consists of a single pore in a lipid bilayer that is created by the oligomerization of the staphylococcal alpha-hemolysin toxin, and a patch clamp amplifier capable of measuring pico Ampere channel currents.

Nanopore Transduction Detection: a highly versatile platform for highly sensitive biosensing

The use of a channel modulator introduces significant, engineered, signal analysis complexity, that we resolve using artificial intelligence (machine learning) methods. The benefit of this complication is a significant gain in sensitivity over T/TD, that often uses a 'sensing' moiety covalently attached to the channel itself, and that typically involves a T/TD-type blockade 'lifetime' event with minimal or no internal blockade structure engineered [44,90]. The NTD approach, on the other hand, has significant improvement in versatility via use of non-covalently 'captured' modulators that can be electrophoretically swapped out on a given channel by voltage reversal. The improvements in sensitivity derive from the measurable, non-trivial, stationary statistics of the channel blockades (and how this can be used to classify state with very high accuracy). The overall improvement in versatility is because all that needs to be redesigned for a different NTD experiment (or binding assay) is the linkage-interaction moiety portion of the bifunctional molecules involved. There is also the versatility that *mixtures* of different types of transducers can be used, a method that can't be employed in single-channel devices that use covalently bound binding moieties (or that discriminate by dwell-time in the channel).

At the nanopore channel one can observe a sampling of bound/unbound states, each sample only held for the length of time necessary for a high accuracy classification. Or, one can hold and observe a single bound/unbound system and track its history of bound/unbound states or conformational states. The *single* molecule detection, thus, allows measurement of molecular characteristics that are obscured in ensemble-based measurements. Ensemble averages, for example, lose information about the true diversity of behavior of individual molecules. For complex *bio*molecules there is likely to be a tremendous diversity in behavior, and in many cases

this diversity may be the basis for their function. The NTD 'Nanoscope' may provide the means to 'see' individual biomolecular kinetics and dynamic behavior. There can also be a great deal of diversity via post-translational modifications, as well, such as with heterogeneous mixtures of protein glycoforms that typically occur in living organisms (e.g., for TSH and hemoglobin proteins in blood serum and red blood cells, respectively). The hemoglobin 'A1c' glycoprotein, for example, is a disease diagnostic (diabetes), and for TSH, glycation is critical component in the TSH-based regulation of the endocrine axis. Multi-component regulatory systems and their variations (often sources of disease) could also be studied much more directly using the NTD approach, as could multi-component (or multi-cofactor) enzyme systems. Glycoform assays, characterization of single-molecule conformational variants, and multi-component assays are significant capabilities to be developed further with the NTD approach, further details on NTD assaying will follow in a later section.

Bifunctional NTD aptamers

In NTD applications we seek DNA modulators with specific, non-linear, topologies, such as Y-shaped DNA duplexes, to obtain molecules whose non-translocating blockades modulate the channel. We include shorter nucleic acids, with channel modulating and simple, DNA-complement, annealing properties, in the collection of DNA-based 'NTD aptamers' described in the NTD biosensor applications that follows. This is because the detection of ssDNA can enable the NTD-transducer's channel-modulatory formation, for direct signal validation, as will be described in what follows.

Potential Impact

Nanopore transduction detection provides an inexpensive, quick, accurate, and versatile method for biosensing, assaying, and performing medical diagnostics. It is hypothesized that NTD biomarkers can be developed for doing the standard clinical tests of the future. The potentially incredible sensitivity and specificity of the NTD targeting on biomarkers also provides a significant new tool for biology, biotechnology, public health and biodefense.

Background

An interdisciplinary perspective is important in understanding the NTD approach. Background is first given on biophysics information flows that result in stationary statistics observations. Then background is provided on the use of stationary statistics signal processing in device enhancement and communication.

Ponderable media flow phenomenology and related information phenomenology

An important part of the NTD methodology is to have a stable ion-channel *sized* such that it can be modulated by a *single*, non-translocating, molecule, where the channel is 'modulated' such that it is significantly blockaded, and not at a fixed level -- e.g., where at least one of the transducer blockade states has multi-level structure (and not simply the approximately Gaussian noise profile of a fixed level blockade). Using a properly-sized channel, it is possible to *establish a modulated ion-flow* through the alpha-hemolysin ion-channel, for example, with a *single* NTD transducer molecule, where the NTD molecule is electrophoretically drawn into the channel.

Biology provides highly stable, nanometer-scale (0.1 -- 100 nm), protein-based ion-channels appropriately sized for the NTD methodology with single-molecule blockaders. A single

molecule's blockading interaction upon capture in an ion channel can be 'self-modulating' upon capture (i.e., without a dominant interaction state), and this has been found in a number of experiments. The mechanism of interaction involves transient chemical bond formation between transducer and protein, where each bound state between transducer and channel imprints on the surrounding ionic current flow to provide a fixed level which then transitions to a different fixed level upon the bond dissociation or transitions to a different bound state. Self-modulatory blockaders each have unique blockade signatures that can be resolved to very high confidence over time. Given the engineering freedom to design the self-modulatory molecules, and the generalizations in the standard periodic carrier based signal processing to stationary statistics carrier based signal processing, we arrive at a means to leverage ponderable media flow phenomena, and blockader interaction kinetics, into a stochastic carrier wave signal processing problem that can be solved using efficient dynamic programming table computational methods as described here.

A single molecule that is captured in an ion channel is not in its natural state. If the captured molecule has been engineered or selected for use in transducing events, then the captured molecule has multiple ion channel flow blockade states. Given the freedom to adjust applied potential, pH, salt, and other factor so as to increase the number or distinctiveness of blockade states, the detector operation typically involves capturing and altering the flow blockaders to produce a biosensing arrangement with flow modulators, or NTD transducers, that responds to specifically designed, or selected, target stimuli (such as binding of the transducer molecule's extra-channel binding moiety region to its target). The captured molecules are altered from their natural form, via stretching and conformational change, in the high electrophoretic force environment at the channel's internal limiting aperture (that prevents the passage of dsDNA). The captured molecules are brought into contact with the channel walls such that chemical interactions take place between portions of the captured molecule and portions of the channel wall, where chemical interactions are taken to include the following binding interactions, among others: electrostatic, pi-bond, ionic, polar covalent, dipole-dipole, hydrogen-bond, Van Der Waals bond, hydrophobic effect bond, and water-of-hydration effect bond.

In a general sense, the nanopore transduction detection method could be broadened to include a detection situation involving functionalization of any flow with stationary statistics, by use of flow modulators that have more than one stationary statistics phase of channel blockade, or flow modulation, where those phases are associated with states of the blockader. Anything that can be 'bathed' in a stationary statistics flow can, thus, be observed by how it modulates that flow. This takes any 'widget' and makes it a 'smart widget' by allowing us to be better informed about its state, and state transitions. All that is required is establishing a stationary statistics flow that is modulated according to state of blockader in some desirable way.

For flow modulation at the nanometer-scale, it is important to note that we are at the scale of single molecules, for which the inherent coherence, and the time-frames for stationary statistics and clearly discernable states (statistical coherence), can be established for greater periods, allowing for greater statistical stability on the modulations produced by a single-molecule interaction (as with high precision atomic clocks being based on a single atom that use a periodic signal attribute in the standard EE signal processing manner).

The lipid bilayer is the main weakness in the protein-channel based approach. Device hardening may eventually be done using solid-state channels, or via hybrid protein channel & 'soft'-state devices (via use of bilayer coat or scaffolding procedures).

Thus, a single molecule's blockading interaction upon capture in an ion channel can be 'self-modulating' upon capture (i.e., without a dominant interaction state), and this has been found in a number of experiments [1-5]. Self-modulatory blockaders each have unique blockade signatures that can be resolved to very high confidence over time. Given the engineering freedom to design the self-modulatory molecules, and the generalizations in the standard periodic carrier based signal processing to stationary statistics carrier based signal processing [6,7], we arrive at a means to leverage ponderable media flow phenomena, and interaction kinetics, into a stochastic carrier wave signal processing problem that can be solved by efficient dynamic programming table computational methods [8,9].

Nanometer-scale flow geometries

At the nanometer-scale of the nanopore experiment, the Reynold's number of the flow is incredibly small (10^{-10}). Thus the flow environment is not fluid-like in a familiar sense. The fluid strongly damps transverse vibrations, for example, so no string-like-motion on polymers occurs. The motions are strongly driven by electrostatic forces and steric constraints and have significant thermal energy contributions, such that a stochastic process is effectively obtained in typical measurements.

In electrochemistry one typically has fluid flow due to the electrophoretic response of simple ions in solution, where a potential is applied across two electrodes immersed in the solution. The electrolytic solution is also the medium for transporting the larger polymers and other solutes. At the scale of single molecules, relevant to nanopore detection, and especially nanopore transduction detection, we find the buffer has structural features. Effects due to water clustering, hydration radius, and Debye radius can be significant. The translocation of neutral polyethylene glycol (PEG) should not have significant enthalpic contribution, for example, but contributions are found [10], where this can be attributed to stripped waters of hydration leading to effective charge sites, and thus stronger enthalpic contributions.

The highly stable, nanometer-scale, α-hemolysin protein channel

The α-hemolysin Nanopore Detector is based on the α-hemolysin transmembrane channel, formed by seven identical 33 kD protein molecules secreted by *Staphylococcus aureus*. The total channel length is 10 nm and is comprised of a 5 nm *trans*-membrane domain and a 5 nm vestibule that protrudes into the aqueous *cis* compartment [28]. The narrowest segment of the pore is a 1.5 nm-diameter aperture [28]. A ring of threonine residues is located at the *cis*-entry. From the *cis*-entry the vestibule diameter opens to approximately 3.6 nm, then shrinks back to the limiting aperture diameter of 1.5 nm. The residues defining the limiting aperture consist of an alternating ring of lysine and glutamate residues. Beyond the limiting aperture is the trans-membrane part of the channel, which consists of neutral residues, except for a ring of leucine residues (hydrophobic). The trans-membrane channel diameter ranges between 1.8 nm and 2.0 nm, and opens at the *trans*-end to a 2.2 nm diameter aperture. The *trans*-side aperture consists of a ring of alternating lysine and aspartate residues (see Fig. 2).

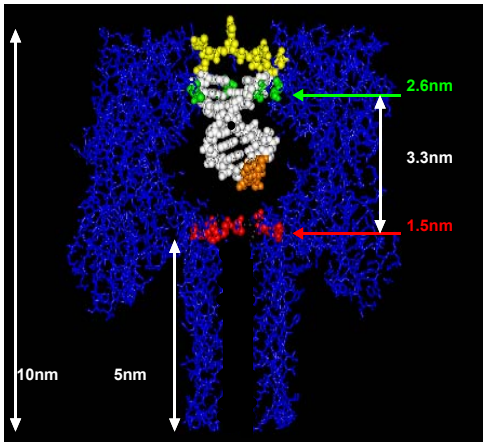

Fig. 2. Alpha Hemolysin Channel, with a nine base-pair DNA hairpin shown captured.

A single strand of DNA is about 1.3 nm in diameter (and an eight nucleotide segment is about 5.4 nm long when fully stretched). Given that water molecules are 0.15 nm in diameter, this means that one hydration layer separates ssDNA from the amino acids in the limiting aperture. This places the charged phosphodiester backbone, hydrogen bond donors and acceptors, and apolar rings of the DNA bases within one Debye length (0.3 nm in 1 M KCl) of the pore wall. Not surprisingly, DNA and RNA strongly interact with the α-hemolysin channel. For applied potentials greater than about 60 mV, however, ssDNA is able to overcome those interactions and is found to translocate. Conclusive evidence describing ssDNA translocation was given by [24], where it was shown that the number of blockade events correlated with the number of translocated ssDNA molecules. Double-stranded DNA, on the other hand, is not found to translocate for any potential. Although dsDNA is too large to translocate, about ten base-pairs at one end can still be drawn into the large cis-side vestibule (see Fig. 2). This actually permits very sensitive experiments, as the ends of "captured" dsDNA molecules can be observed for extensive periods of time to resolve features [2,4,5].

The alpha-Hemolysin nanopore blockade detector

Nanopore detection is based on a nanometer-scale ion channel that can report on the channel-interactions of individual, nanometer-scale, biomolecules. The reporting is via measurements of ion flow through the channel when there is typically only a single channel, i.e., there is only one conductance path [1,2,5,19,23,24,36-38].

Each experiment described in what follows was conducted using one alpha-hemolysin channel inserted into a diphytanoyl-phosphatidylcholine/hexadecane bilayer, where the bilayer was formed across a 20-micron diameter horizontal Teflon aperture [1] (see Fig. 3). The bilayer separates two seventy-microliter chambers containing 1.0 M KCl buffered at pH 8.0 (10 mM HEPES/KOH). A completed bilayer between the chambers was indicated by the lack of ionic current flow when a voltage was applied across the bilayer (using Ag-AgCl electrodes). Once the bilayer was in place, a dilute solution of α–hemolysin (monomer) was added to the *cis* chamber. Self-assembly of the α–hemolysin heptamer and insertion into the bilayer results in a stable, highly reproducible, nanometer-scale channel with a steady current of 120 pA under an applied potential of 120 mV at 23°C (± 0.1 °C using a Peltier device). Once one channel formed, further pores were prevented from forming by thoroughly perfusing the *cis* chamber with buffer. Molecular blockade signals were then observed by mixing analytes into the *cis* chamber. The

experiment is performed on a vibration isolation table to keep the bilayer from rupturing (and ending the experimental setup). Synthetic S-layer scaffolding and other bilayer-strengthening methods may eventually eliminate the need for vibration isolation.

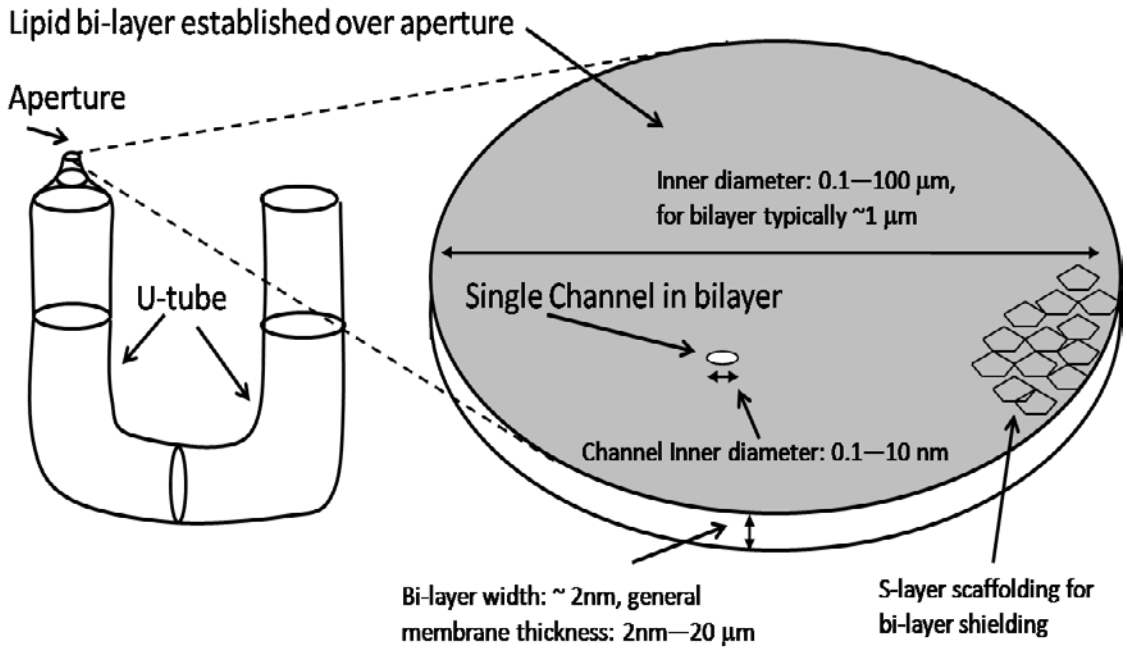

Fig. 3. A schematic for the U-tube, aperture, bilayer, and single channel, with possible S-layer modifications to the bi-layer.

Single biomolecules, and the ends of biopolymers such as DNA, have been examined in solution with nanometer-scale precision using nanopore blockade detection [1,2,5,25]. In early studies [1], it was found that complete base-pair dissociations of dsDNA to ssDNA, "melting", could be observed for sufficiently short DNA hairpins. In later work [2,5], the nanopore detector attained Angstrom resolution and was used to "read" the ends of dsDNA molecules. In [4,25,39], the nanopore detector was used to observe the conformational kinetics of the end regions of individual DNA hairpins.

The five DNA hairpins shown in Fig. 4 have been studied in [2,5], where they have been carefully characterized, and are used in other experiments as controls. Use of the controls entails testing a channel, especially an oddly behaving channel, with a known nine base-pair DNA hairpin control. If the familiar, visibly discernible, control blockade signals do not occur, the channels viability is then looked into further. The nine base-pair hairpin molecules examined in the prototype experiment share an eight base-pair hairpin core sequence, with addition of one of the four permutations of Watson-Crick base-pairs that may exist at the blunt end terminus, i.e., 5'-G•C-3', 5'-C•G-3', 5'-T•A-3', and 5'-A•T-3'. Denoted 9GC, 9CG, 9TA, and 9AT, respectively. The full sequence for the 9CG hairpin is 5' CTTCGAACGTTTT CGTTCGAAG 3', where the base-pairing region is underlined. The eight base-pair DNA hairpin is identical to the core nine base-pair subsequence, except the terminal base-pair is 5'-G•C-3'. The prediction that each hairpin would adopt one base-paired structure was tested and confirmed using the DNA mfold server.

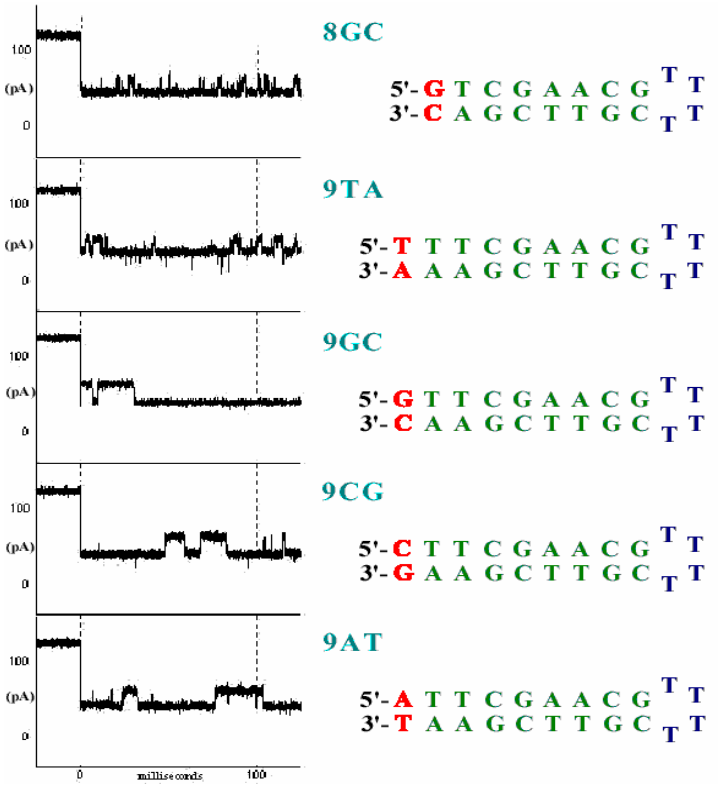

Figure 4. DNA hairpin controls and their diagnostic signals. The secondary structure of the DNA hairpins is shown on the right, with their highest scoring diagnostic signals shown on the left [5]. Each signal trace starts at approximately120 pA open channel current and all blockades are in a range 40-60 pA upon "capture" of the associated DNA hairpin. Even so, the signal traces have discernibly different blockade structure, which is extracted using an HMM. The signals are aligned at their blockade starts and the demarked time-trace is for 100 ms.

Summary of Nanopore Detector based biosensing methods

The standard Nanopore Detector (ND) detection paradigm, that is predominantly translocation (or dwell-time) based, is shown in Fig. 5 side-by-side with the Nanopore *transduction* detector paradigm. Figure 6 elaborates on the possible ND detection platform topologies possible with translocation-based approaches, where the difference in translocation times is often the critical information that is used. The difference in dwell times can depend on the off-binding time of the target binding entity (possibly in a high strain environment), where binding failure allows polymer (ssDNA) translocation to complete (and the channel blockade to end). By this mechanism, and its variants, bound probes can be distinguished from unbound. There are specificity limits on the melting-based detection, however, that are not a problem in the NTD approach.

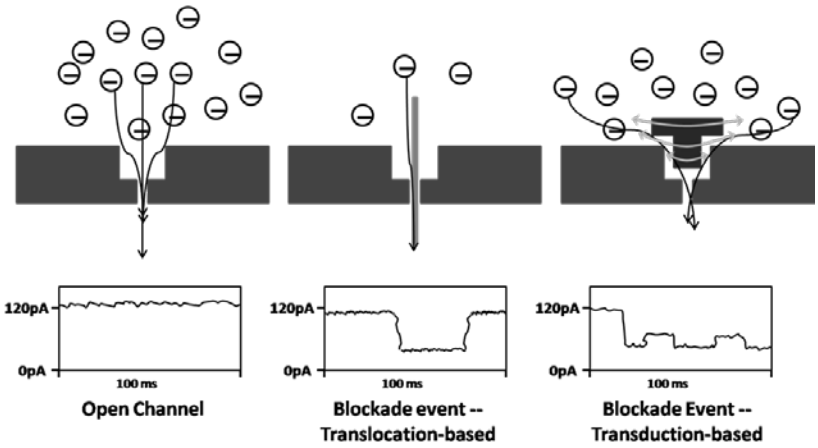

Fig. 5. Translocation Information and Transduction Information . Left. Open Channel . **Center.** A channel blockade event with feature extraction that is typically dwell-time based. A Single-molecule coulter counter. **Right.**

Single-molecule transduction detection is shown with a transduction molecule modulating current flow (typically switching between a few dominant levels of blockade, dwell time of the overall blockade is not typically a feature -- many blockade durations will not translocate in the time-scale of the experiment, for example, active ejection control is often involved).

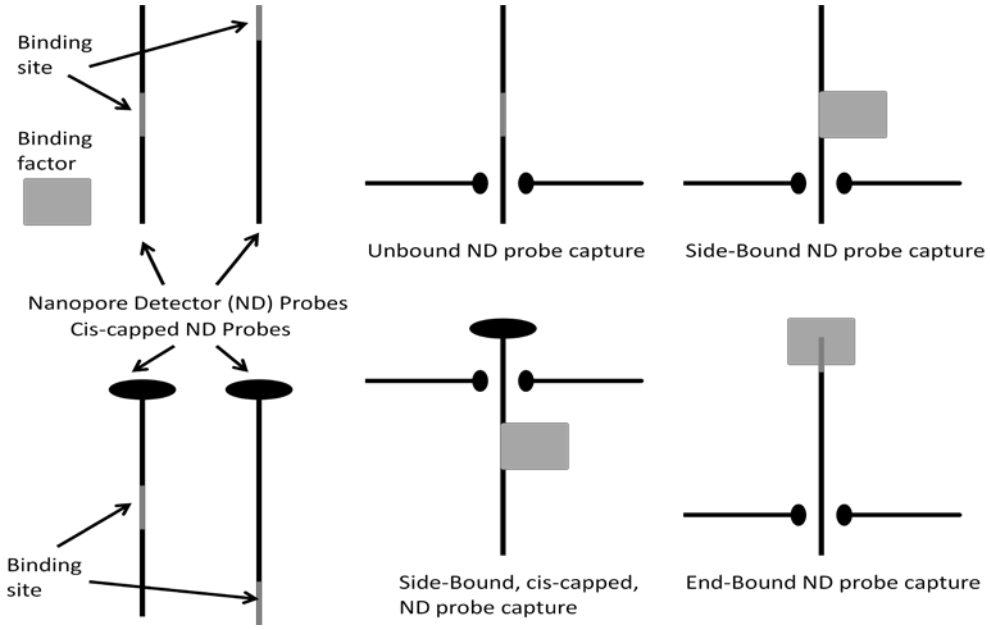

Fig. 6. Nanopore Detector detection topologies involving polymer translocation or threading. The detection event is given by polymer (ssDNA in [45-64]) translocations that are delayed if bound (side and end configurations shown). If bound entity is on the trans side (with cis-side capped, or vice versa), and bound entity is a processive DNA enzyme, then sequencing may be possible as described in [53,65-68].

Nanopore Transduction Detection Methods and Results

NTD Biosensing Methods & Proof of Concept Results

NTD biosensing methods can involve a DNA modulator with linkages to an aptamer, antibody, or some other binding moiety, including simply a ssDNA overhang. The linkages needed to connect a DNA-based channel-modulator to a DNA-based aptamer involves a trivial join of the underlying ssDNA sequences involved. The linkage needed to connect a DNA-based channel-modulator to an antibody *could* involve use of linker technology, and this has been used in the past with dsDNA hairpins [88], but another, more commoditized route, easily accessible with use of the NADIR refined Y-shaped DNA channel modulators [3,87], is that the antibody need merely be 'tagged' with the appropriate ssDNA strand, e.g., where the DNA sequence is complement to part of the 'Y' shaped DNA channel modulator, and antibody tagging with DNA is a standard service for use in immuno-PCR. Proof-of-Concept Experiments are described next for the streptavidin-biotin and DNA annealing model systems, a pathogen/SNP detection prototype, and for aptamer and antibody based detection.

Model system based on streptavidin and biotin

A biotinylated DNA-hairpin is engineered to generate two signals depending on whether or not a streptavidin molecule is bound to the biotin (see Figs. 8 & 9). Results (from [83] in Fig. 8 (Right) suggest that the new signal class on binding is actually a racemic mixture of two hairpin-loop twist states. At T=4000 urea is introduced at 2.0 M and gradually increased to 3.5 M at T=8,100.

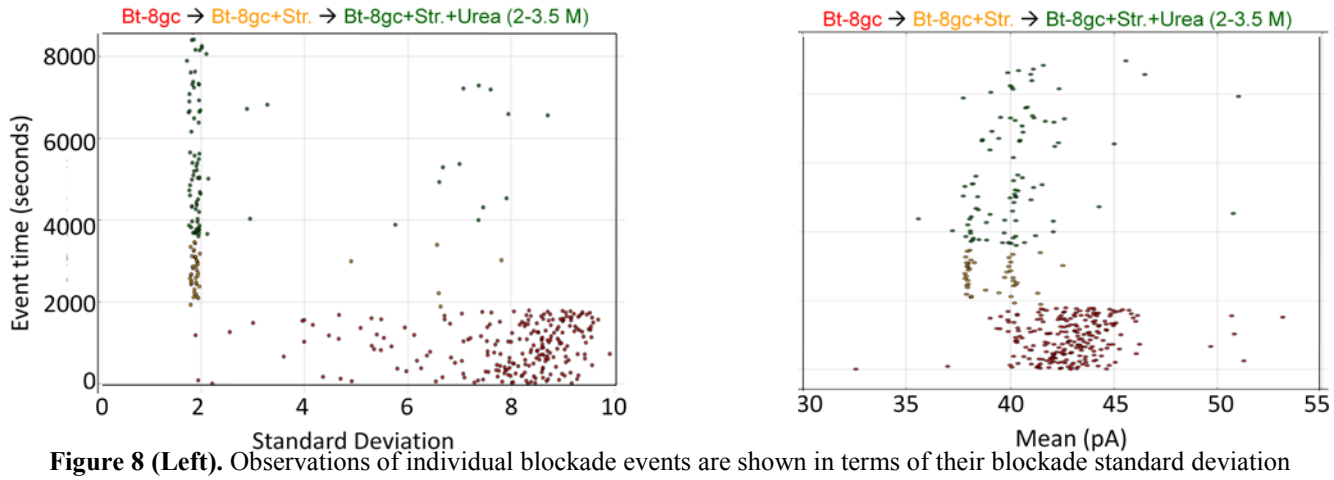

Figure 8 (Left). Observations of individual blockade events are shown in terms of their blockade standard deviation (x-axis) and labeled by their observation time (y-axis). The standard deviation provides a good discriminatory parameter in this instance, since the transducer molecules are engineered to have a notably higher standard deviation than typical noise or contaminant signals. At T=0 seconds, 1.0 µM Bt-8gc is introduced and event tracking is shown on the horizontal axis via the individual blockade standard deviation values about their means. At T=2000 seconds, 1.0 µM Streptavidin is introduced. Immediately thereafter, there is a shift in blockade signal classes observed to a quiescent blockade signal, as can be visually discerned. The new signal class is hypothesized to be due to (Streptavidin)-(Bt-8gc) bound-complex captures. **(Right)**. A marked change in the Bt-8gc blockade observations is shown immediately upon introducing streptavidin at T=2000 seconds, but with the mean feature we clearly see two distinctive and equally frequented (racemic) event categories. Introduction of chaotropic agents degrades first one, then both, of the event categories, as 2.0 M urea is introduced at T=4000 seconds and steadily increased to 3.5 M urea at T=8100 seconds.

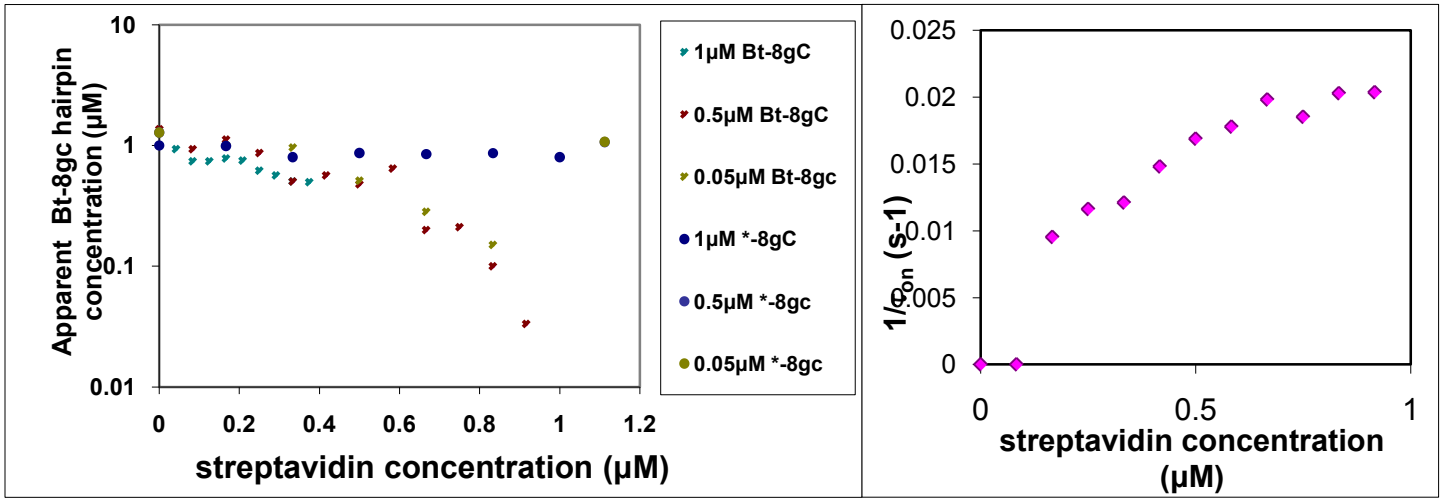

Figure 9 (Left). The apparent Bt-8gc concentration upon exposure to Streptavidin. The vertical axis describes the counts on unbound Bt-8gc blockade events and the above-defined mapping to "apparent" concentration is used. In the dilution cases, a direct rescaling on the counts is done, to bring their "apparent" concentration to 1.0 µM concentration (i.e., the 0.5 µM concentration counts were multiplied by 2). For the control experiments with no biotin (denoted '*-8gc'), the *-8gc concentration shows no responsiveness to the streptavidin concentration. **(Right). The increasing frequency of the blockades of a type associated with the streptavidin-Bt-8gc bound complex.** The background Bt-8gc concentration is 0.5 µM, and the lowest clearly discernible detection concentration is at 0.17 µM streptavidin.

Figure 9 (details in [83]) shows transduction of bound/unbound signals at three different transducer concentrations and a range of binding target (streptavidin) concentrations. Rescaling is done on counts, with the count of events at the 0.05 μM concentration Bt-8gC scaled up by a factor of 20, for example, for comparing event rate observations at different concentrations with the 1 μM Bt-8gC behavior (where a linear response with concentration will result in an overlay of the different plots according to class, as is seen over a two-magnitude range). Thousands of individual blockade observations are used to obtain the observation counts, and from this the 'apparent concentration' at the various streptavidin concentrations. The good agreement of the curves in Fig. 9 strongly validates the NTD biosensing hypothesis and indicates a linear response where probe density can also provide a detector sensitivity gain in circumstances where pattern-recognition informed nanopore sampling/selection is enabled to effectively 'ignore' unbound probes.

Model system based on DNA annealing

A unique, Y-shaped, NTD-aptamer is described in Fig. 10. In this experiment a stable modulator is established using a Y-shaped molecule, where one arm is loop terminated such that it can't be captured in the channel, leaving one arm with a ssDNA extension for annealing to complement target.

A preliminary test of DNA annealing has been performed with the Y-shaped DNA transduction molecule indicated, where the molecule is engineered to have an eight-base overhang for annealing studies. A DNA hairpin with complementary 8 base overhang is used as the binding partner. Figure 11 shows the binding results at the population-level (where numerous single-molecule events are sampled and identified), where the effects of binding are discernible, as are potential isoforms, and the introduction of urea at 2.0 M concentration is easily tolerated (a mild chaotrope) and actually helps in discerning collective binding interactions such as with the DNA annealing.

Only a portion of a repetitive validation experiment involving the molecules in Fig. 10 is shown in Fig. 11, thus time indexing starts at the 6000th second. From time 6000 to 6300 seconds (the first 5 minutes of data shown) only the DNA hairpin is introduced into the analyte chamber, where each point in the plots corresponds to an individual molecular blockade measurement. At time 6300 seconds urea is introduced into the analyte chamber at a concentration of 2.0 M. The DNA hairpin with overhang is found to have two capture states (clearly identified at 2 M urea).

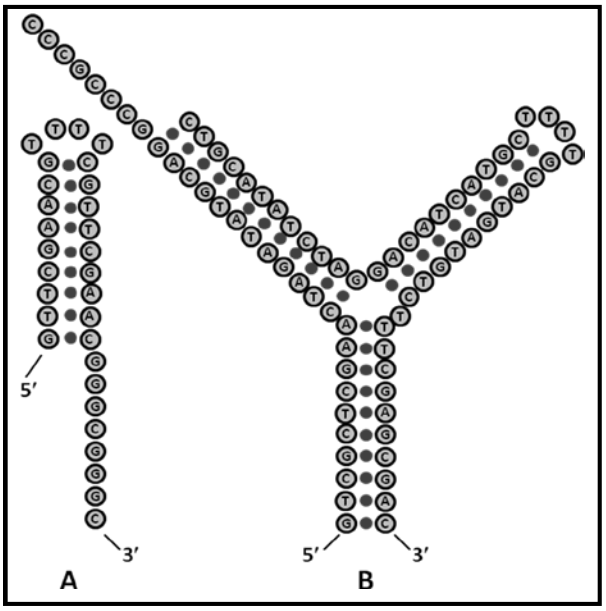

Figure 10. The Y-Anneal transducer, and its annealing target.

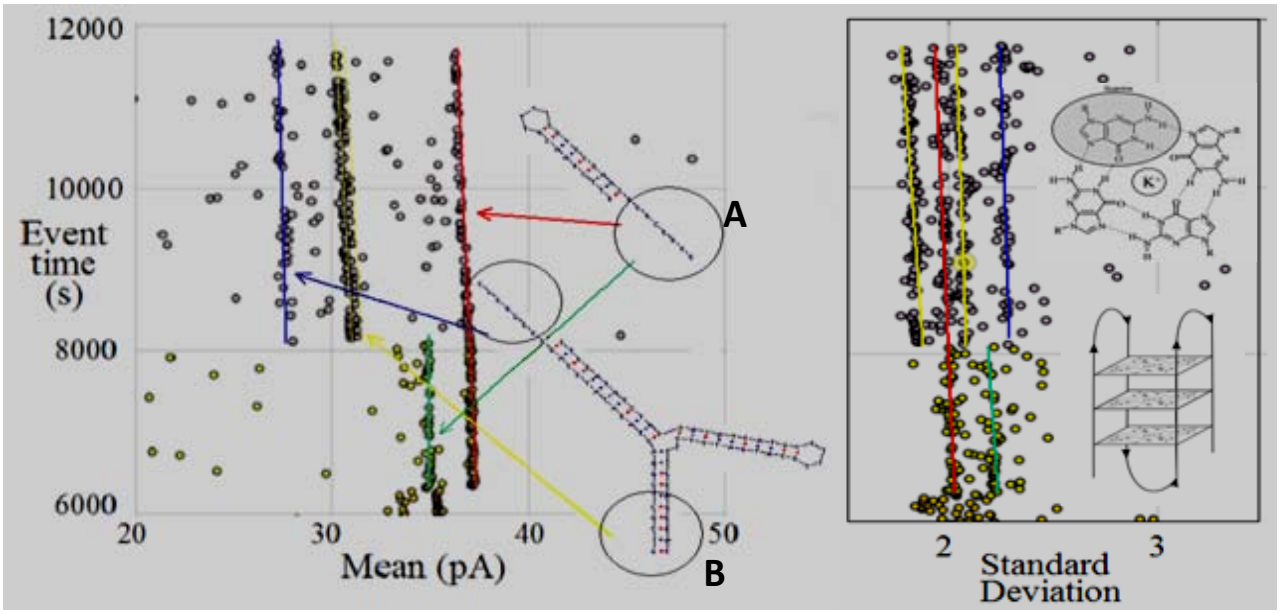

Figure 11. Y-shaped DNA transducer with overhang binding to DNA hairpin with complementary overhang. The two hairpin channel-capture states are marked with the green and red lines, in both the plot of signal means and signal standard deviations. After 30 minutes of sampling on the hairpin+urea mixture (from 6300 to 8100 seconds), the Y-shaped DNA molecule is introduced at time 8100. Observations are shown for an hour (8100 to 11700 seconds). A number of changes and new signals now are observed: (i) the DNA hairpin signal class identified with the green line is no longer observed – this class is hypothesized to be no longer free, but annealed to its Y-shaped DNA partner; (ii) the Y-shaped DNA molecule is found to have a bifurcation in its class identified with the yellow lines, a bifurcation clearly discernible in the plots of the signal standard deviations. (iii) the hairpin class with the red line appears to be unable to bind to its Y-shaped DNA partner, an inhibition currently thought to be due to G-quadruplex formation in its G-rich overhang. (iv) The Y-shaped DNA molecule also exhibits a signal class (blue line) associated with capture of the arm of the 'Y' that is meant for annealing, rather than the base of the 'Y' that is designed for channel capture. In the Std. Dev. box are shown diagrams for the G-tetrad (upper) and the G-quadruplex (lower) that is constructed from stacking tetrads. The possible observation of G-quadruplex formation bodes well for use of aptamers in further efforts.

Preliminary SNP Detection Efforts

A possible test of DNA SNP annealing is with the Y-shaped DNA transduction molecule shown in Fig. 10('B') that is minimally altered, and such that the SNP variant occurs in the Y-nexus region. For the case where digestion can't conveniently provide extension only to one-side, a Y-shaped annealed dsDNA molecule can still be obtained, but such that the ssDNA extensions outside the annealed region are now free to extend on both arms of the Y-molecule.

SNP variant detection is reduced to resolving the signals of two Y-shaped duplex DNA molecules, one with mismatch at SNP, one with Watson-Crick base-pairing match at SNP. In preliminary studies of Y-shaped DNA molecules, numerous Y-shaped DNA molecules were considered. Three variants that successfully demonstrated the easily discernible, modulatory, channel blockade signals are shown in Fig. 12 [87]. In those variants we considered the Y-nexus with and without an extra base (that is not base-paired). If an extra base is inserted we explore the three positions at the Y (left and middle inserts shown in the left and center Y-molecules shown in Fig. 12.

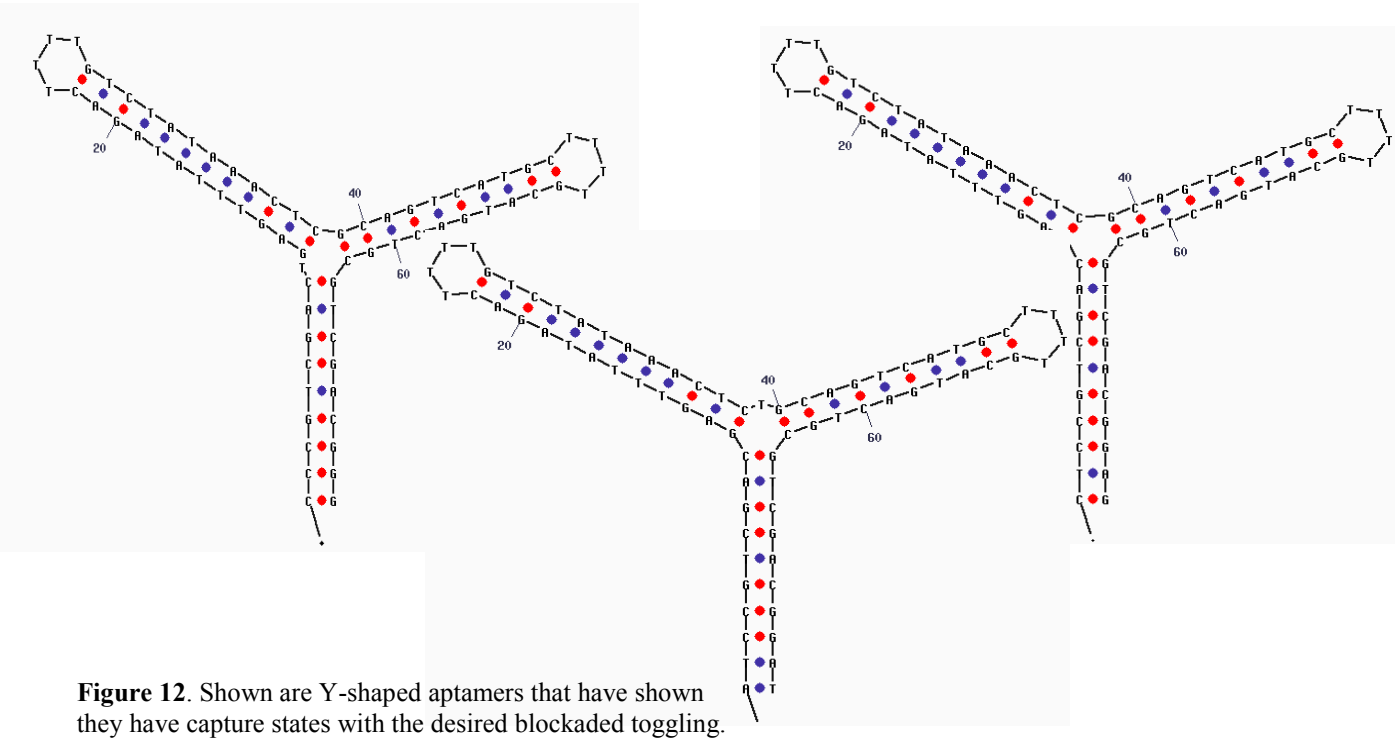

Figure 12. Shown are Y-shaped aptamers that have shown they have capture states with the desired blockaded toggling.

The DNA molecular design used in [87] consists of a three-way DNA junction created: 5'-CTCCGTCGAC GAGTTTATAGAC TTTT GTCTATAAACTC GCAGTCATGC TTTT GCATGACTGC GTCGACGGAG-3'. Two of the junctions' arms terminate in a 4T-loop and the remaining arm, of length 10 base-pairs, is usually designed to be blunt ended (sometimes shorter with an overhang). The blunt ended arm has to be carefully designed such that when it is captured by the nanopore it produces a toggling blockade. One of the arms of the Y-shaped aptamer (Y-aptamer) has a TATA sequence, and is meant to be a binding target for TBP. In general, any transcription factor binding site could be studied (or verified) in this manner. Similarly, transcription factor could be verified by such constructions, or the efficacy of a synthetic transcription factor could be examined.

In using the NADIR refinement process to arrive at the Y-transducer used in the DNA annealing test [87], we have demonstrated how *single-base insertions or modifications at the nexus of the Y-shaped molecule can have clearly discernible changes in channel-blockade signal*. Y-molecules as DNA probes with single point mutations discernible at the Y-nexus are explored in [87] (see Fig. 12). What is described in [87] is a linkage to a *na*nopore-detector *dir*ected (NADIR) search for aptamers that is based on bound-state lifetime measurements (or some other selection criterion of interest). NADIR complements and augments SELEX in usage. Further discussion of NTD Biosensing experiments along these lines is in the Discussion, for SNP, Aptamer, and Antibody based detection systems. Extensive further results in these areas are described in [99], as well.

NTD Assaying Methods and Results

Using a NTD 'Nanoscope', a single bound/unbound system can be held, observed, and its history of bound/unbound states can be tracked. The *single* molecule state-tracking with lengthy time averages allows measurement of molecular characteristics that are obscured in ensemble-based measurements. The ensemble averages that underlie most approaches, lose information about the true diversity of behavior of individual molecules. For complex biomolecules there is likely to be a tremendous diversity in behavior, and in many cases this diversity may be the basis for their function.

DNA enzyme analysis: Integrase

DNA termini are of critical importance for certain retroviral integrases and other biological processes – being able to study them, even comparatively, offers new avenues for understanding and drug selection (HIV integrase blockers). Information on the DNA molecules' variation in structure and flexibility is important to understanding the dynamically enhanced (naturally selected) DNA complex formations that are found with strong affinities to other, specific, DNA and protein molecules. An important example of this is the HIV attack on cells. The DNA terminus properties of retroviral DNA molecules are found to exhibit greater flexibility than similar sequences, often marked by an increase in the number of blockade states, such as in the upper-level fine structure for the molecule terminating with GACG-3' [40].

One of the most critical stages in HIV's attack is the binding between viral and human DNA. The DNA molecule studied in this instance consists of the HIV consensus terminus at the end of the Y-aptamer arm – where it is exposed for binding to integrase. Since this molecule presents another blunt-ended dsDNA for capture, it is no surprise that such events occur. The signal analysis must separate between two classes of signal associated with these two dominant forms of capture -- associated with capture of the two blunt-ended DNA regions (at the base of the Y and at the end of the integrase-binding arm). With appropriate capture of the molecule at the base of the Y, this permits direct examination of protein binding to the terminal DNA region.

The NTD approach may provide the best means for examining other enzymes, and other complex biomolecules, particularly their activity in the presence of different co-factors. There are two ways that these studies can be performed: (i) the enzyme is linked to the channel transducer, such that the enzyme's binding and conformational change activity may be directly observed and tracked or, (ii) the enzyme's substrate may be linked to the channel transducer and observation of enzyme activity on that substrate may then be examined. Case (i) provides a means to perform DNA sequencing if the enzyme is a nuclease, such as lambda exonuclease. Case (ii) provides a means to do screening, for example, against HIV integrase activity (for drug discovery on HIV integrase inhibitors). Further details can be found in [100].

Single-molecule serial assaying

Preliminary work assaying glycoproteins has mainly focused on the HbA1c glycoform assayer for patient glucose-level testing. Initial results indicate a good tolerance for the typical contaminants present in blood, including high concentrations of hemoglobin itself. Further details on efforts in-progress are in the Discussion.

Channel Current Cheminformatics (CCC) Methods

Fig. 21 shows the pattern recognition informed signal processing architecture [4,5,37,78]. The processing is designed to rapidly extract useful information from noisy blockade signals using feature extraction protocols, wavelet analysis, Hidden Markov Models (HMMs) and Support Vector Machines (SVMs). For blockade signal acquisition and simple, time-domain, feature-extraction, a Finite State Automaton (FSA) approach is used [79] that is based on tuning a variety of threshold parameters. A generic HMM is then used to characterize current blockades by identifying a sequence of sub-blockades as a sequence of state emissions [5,80,81]. The parameters of the generic-HMM can then be estimated using a method called Expectation/Maximization, or 'EM" [80], to effect de-noising. The HMM method with EM is part of the standard implementation used in what follows. Classification of feature vectors obtained by the HMM for each individual blockade event is then done using SVMs. For the nanopore detector augmented with auxiliary molecules, much more data is usually needed to properly train the Machine Learning algorithms. The distributed training of these algorithms (recently established in [82]) is a critical component in real-time signal processing [37,78]. The CCC software helps in the discovery, characterization, and classification of localizable, approximately-stationary, statistical signal structures in channel current data, and changes between such structures. Along the lines of previous work in channel current cheminformatics [1,2,4,5,81,25], the CCC real-time implementation is used for analysis of the data to be measured, and refined as needed.

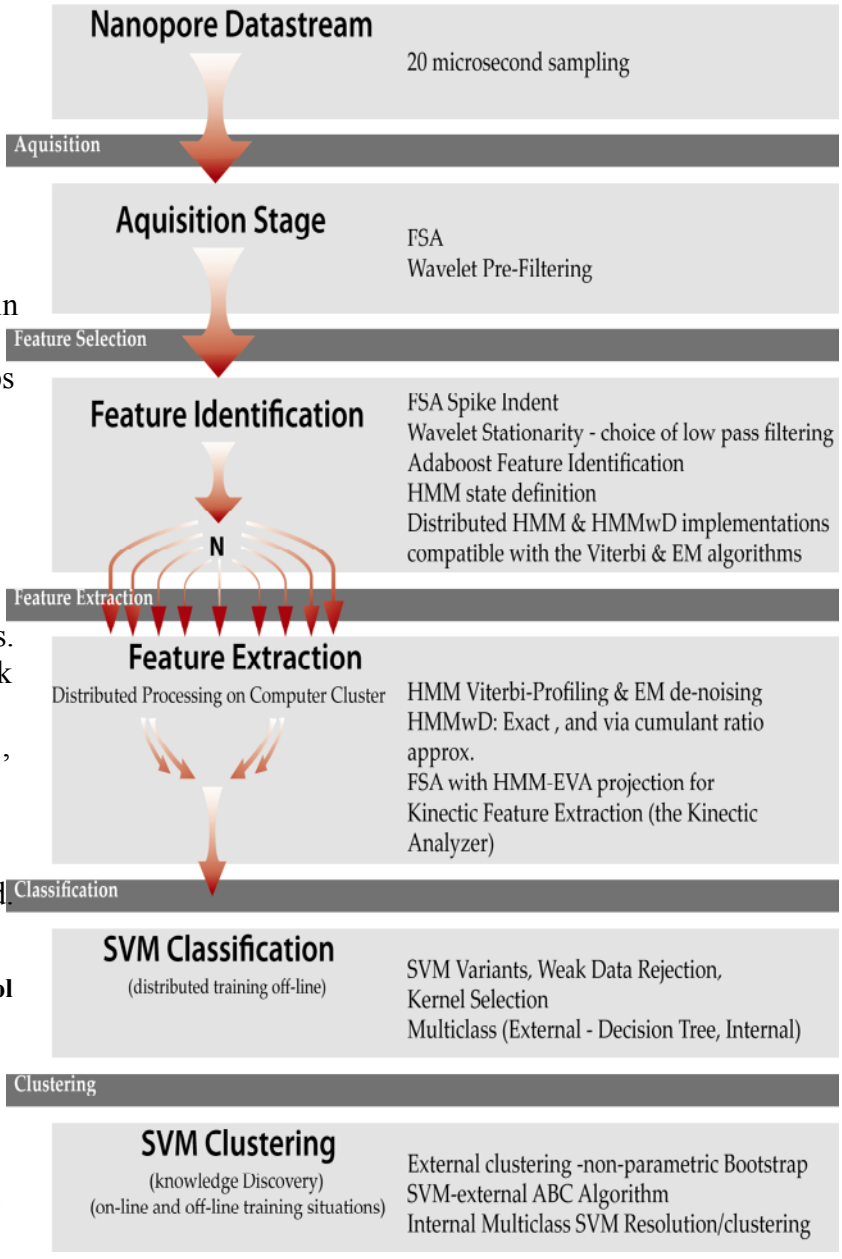

Figure 21. Nanopore cheminformatics & data-flow control architecture. Aside from the modular design with the different machine learning methods shown (HMMs, SVMs, etc.), recent augmentations to this architecture for real-time processing include use of a LabWindows Server to directly link to the patch-clamp amplifier.

Discussion

Prototype NTD based Detection Systems

SNP Detection

A preliminary test of DNA SNP annealing can be done with the Y-shaped DNA transduction molecule shown in Fig. 13, which is minimally altered (e.g., mostly common sequence identity) from the Y-annealing transducer studied in Fig. 12.

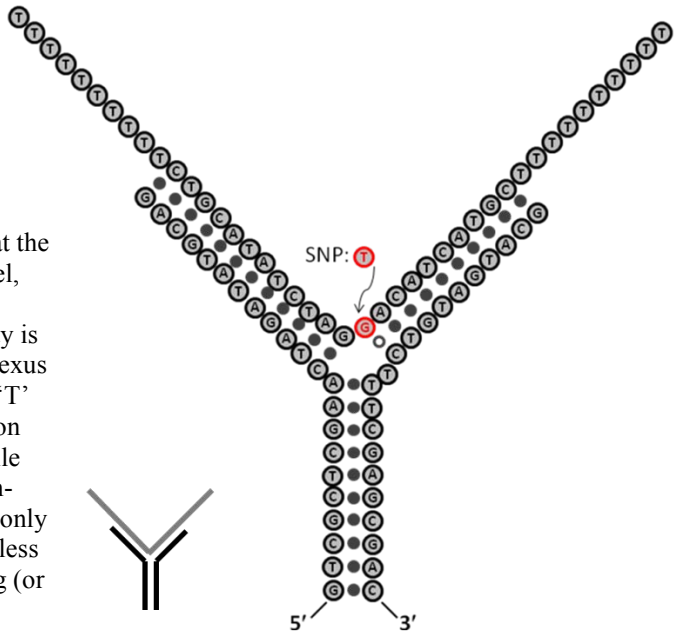

Figure 13. The Y-SNP with test complex is shown at the base-level specification and at the diagrammatic level, where a SNP base is as indicated. If the SNP is its variant form (typically only one other base possibility is common), then a base-pairing will not occur at the nexus of the Y-SNP shown (with the red base becoming a 'T' in the variant as indicated). This allows discrimination between the annealed forms with high accuracy, while also discerning from the signals produced by the non-annealed Y-SNP, where there is no target-bound, or only non-specific molecular interactions imparting much less conformational structure as occurs with the matching (or mostly matching) annealing interaction.

Once the Y-SNP transducer has been tested on a single-species of short overhang length test molecules the next experimental challenge will be to detect SNP variants using the Y-SNP transducer probe in the presence of a heterogeneous length mixture (some with target SNP region of interest), with overhang as shown in Figure 14.

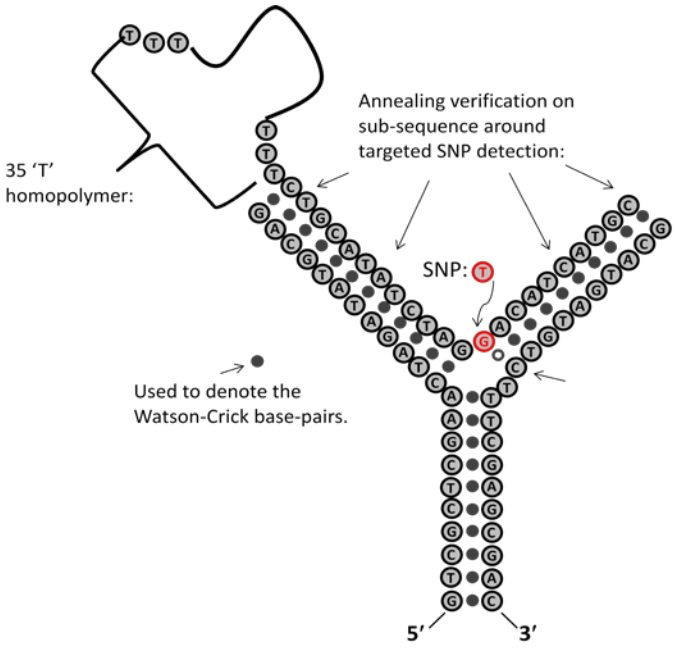

Figure 14. The Y-SNP test complex with 35 dT length overhang is shown at the base-level specification, where a SNP base is as shown. If the SNP is its variant form (typically only one other base possibility is common), then a base-pairing will not occur at the nexus of the Y-SNP shown. This allows discrimination between the annealed forms with high accuracy, while also discerning from the signals produced by the non-annealed Y-SNP, where there is no target-bound, or only non-specific molecular interactions imparting much less conformational structure as occurs with the matching (or mostly matching) annealing interaction.

The value of 35 ‘T’s on the extension is to also match the approximate extension, with the same ‘Y’-sequence (except for a 4 dT cap) as the previously ‘blunt-ended’ annealed conformation. SNP variant detection is reduced to resolving the signals of two Y-shaped duplex DNA molecules, one with mismatch at SNP, one with Watson-Crick base-pairing match at SNP. From the above, it is clear that the NTD method provides a viable prospect for SNP variant detection to very high accuracy (possibly the accuracy with which the NTD can discern DNA control hairpins that only differ in terminal base-pair). SNP detection via *translocation-based* methods, on the other hand, must discern between two SNP variants according to the different dwell times of the complement-template annealed SNPs, until dissociation from the template allows translocation of the blockading dsDNA annealed conformation.

Aptamer-based Detection

Aptamers are especially appropriate for study by nanopore detection due to the fact they can be designed with an end to be captured and modulate a nanopore (i.e., the captured end is dsDNA), while other parts of the aptamer are intended to bind a specific target. This directly provides a NTD transducer if one or both of the bound/unbound states (captured in the channel at the dsDNA end) provides distinctive channel modulations. The binding statistics derived from the study of aptamers in a nanopore detector can also be used in the design of the aptamer itself, e.g., NADIR selection instead of further SELEX-based selection [87]. In Figure 15 we see the first aptamer test case to be considered, where we seek to detect thrombin [93] in one case, and IgG [94] in another. In one effort, we use the thrombin aptamer found by Ikebukuro et al [93], which is selected via SELEX and EMA and is a 31-mer,that we link by a 4 dT spacer to the Y-transducer (see Fig. 15).

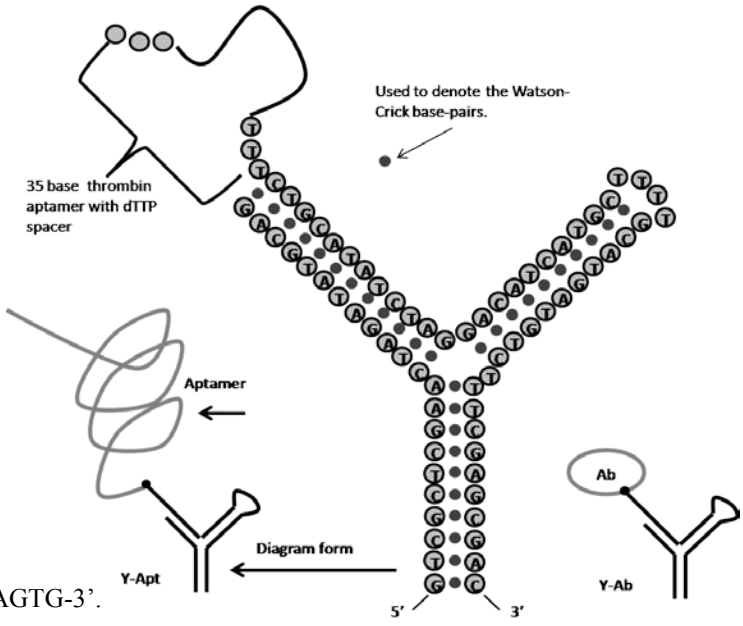

Fig. 15 The thrombin aptamer from [93] is 5’-CACTGGTAGGTTGGTGTGGTTGGGGCCAGTG-3’.

Antibody-based Detection

Linkage of ssDNA to antibody is commonly done in immuno-PCR preparations, so another path with rapid deployment is to make use of a linkage technology that is already commoditized, e.g., the molecules required for the antibody-based biosensing with this approach are simple (non-specialty) molecular components. The core issue to be resolved here is whether a good NTD

signal can be produced with immuno-PCR tagged antibodies that are designed to anneal to another DNA molecule to form an NTD 'Y-transducer' (see Fig. 15, lower right). From previous efforts [88], with more complicated EDC linkages between a modified thymine and an antibody, it is clear that there are strong prospects for success with this method. What is sought is not just further validation of the method, however, but a less expensive, accessible, platform from which to refine and develop NTD-based systems.

Prototype NTD based Assaying Systems

The NTD Glycoprotein assayer

NTD can operate as an HbA1c glycoform assayer to improve the knowledge of hemoglobin biochemistry (and that of heterogeneous, transient, glycoproteins in general). This could have significant medical relevance, as a gap exists between what is known about hemoglobin biochemistry and how HbA1c information is used in the management of diabetic patients. The definition of 'HbA1c' is complex, as HbA1c is a heterogeneous mixture of non-enzymatically modified hemoglobin molecules (whose concentration in blood is in part genetically determined). In clinical applications, HbA1c is used as if it were single complex with glucose whose concentration is solely influenced by glucose concentration. It may be possible, using an NTD platform, to improve diabetes management by introducing a new assaying capability to directly close the gap between the basic and clinical knowledge of HbA1c. It may be possible, perhaps optimal, to apply NTD in direct nanopore detector-to-target assays, in combination with indirect NTD-to-target assays, for purposes of characterizing post-translational protein modifications (glycations, glycosylations, nitrosilations, etc.), see Fig. 16.

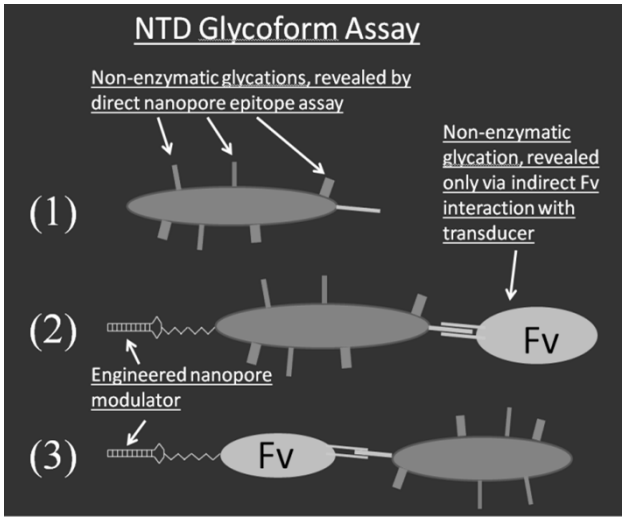

Figure 16. NTD-based glycoform assays. Three NTD Glycoform assays are shown. Assay method (1) shows a protein with its post-translational modifications in orange (e.g., non-enzymatics glycations, glycosylizations, advanced glycation end products, and other modifications). Assay method (2) shows a protein of interest linked to a channel modulator. Direct channel interactions (blockades) with the protein modifications are still possible in this instance, but are expected to be dominated by the preferential capture of the more greatly charged modulator capture. Changes in that modulator signal upon antibody Fv interactions with targeted surface features provide an indirect measure of those surface feature. Assay method (3) shows an antibody Fv that is linked to modulator, where, again, a binding event is engineered to be transduced into a change of modulator signal.

The endocrine axis, thyroid stimulating hormone (TSH) in particular, is regulated via a heterogenous mixture of TSH molecules with different amounts of glycation (and other modifications). The extent of TSH glycation is a critical regulatory feedback mechanism. Tracking the heterogenous populations of critical proteins is critical to furthering our understanding and diagnostic capabilities for a vast number of diseases. Hemoglobin molecules

provide a specific, on-the-market, example -- here extensive glycation is more often associated with disease, where the A1c hemoglobin glycation test is typically what is performed in many over-the-counter blood monitors. The NTD testing of surface features of the protein can be done before or after digestion or other modification of the test molecule as a means to further improve signal contrast on the identity and number of possible protein modifications, as well as other surface features.

Part of the complexity of glycoforms, and other modifications, of proteins such as hemoglobin and TSH, is that these glycoforms are present as a heterogeneous mixture, and it is the relative populations of the different glycoforms that may relate to clinical diagnosis or identification of disease (such as prion exposure [98]). To this end, a protein's heterogeneous mixture of glycations and other modified forms can be directly observed with a NTD nanoscope, and this constitutes the clinically relevant data of interest, not simply the concentration of some particular glycoform. Furthermore, it is the transient, dynamic, changes of the glycoform profile that is often the data of interest, such that a 'real-time' profile of glycoform populations may be of clinical relevance, and obtaining such real-time profiling of modified forms (glycoforms, etc.) would be another area of natural advantage for the NTD approach.

Protein modification assays have indirect relevance for public health and biodefense. This is because the degree of glycation of a patients hemoglobin is an early indication of their disease state (if any, or simply 'glycation' age otherwise). This is because the *hemoglobin that is actively used in transporting oxygen throughout the body is analogous to a 'canary- in-the-coalmine' in that it provides an early warning about insipient complications or past chemical or nerve agent exposures.* Red blood cells (that carry hemoglobin) typically live for 120 days – providing a 120-day window into past exposures and a 120-day average on the regulatory load induced by those exposures. In the future, if a mysterious gulf-war syndrome is encountered, and there is concern about a low-level exposure to a nerve agent, examining the hemoglobin glycation profiles, and similar profiles on other blood serum constituents, would provide a rapid (30 min.) assessment of biodefense status.

NTD detection and assaying provides a new technology for characterization of transient complexes, with a critical dependence on 'real-time' cyberinfrastrucure that is integrated into the nanopore detection method, using machine learning methods for pattern recognition, and their implementation on a distributed network of computers for real-time experimental feedback and sampling control.

Antibody Assayer

Upon binding to antigen, a series of events are initiated by the interaction of the antibody carboxy-terminal region with serum proteins and cellular receptors. Biological effects resulting from the carboxy-terminal interactions include activation of the complement cascade, binding of immune complexes by carboxy-terminal receptors on various cells, and the induction of inflammation. Nanopore Detection provides a new way to study the binding/conformational histories of individual antibodies. Many critical questions regarding antibody function are still unresolved: questions that can be approached in a new way with the nanopore detector. The different antibody binding strengths to target antigen, for example, can be ranked according to the observed lifetimes of their bound states. Questions of great interest include: are allosteric

changes transmitted through the molecule upon antigen binding? Can effector function activation be observed and used to accelerate drug discovery efforts?

Thus, real-time analysis of antibody IgG binding affinity may be possible using a nanopore detector to better understand antibody-antigen binding affinities and the conformational changes that initiate signal pathways. Although some surface features clearly elicit blockade signals that are modulatory, not all surface features of interest will exhibit blockade signals when drawn to the channe,l and in these instances antibody or aptamer based targeting of those features could be used, where the antibody or aptamer is linked to a channel modulator that then reports on the presence of the targeted surface feature indirectly, e.g., the NT-biosensing setup.

A nanopore-based glycoform assay could be performed on modified forms of the proteins of interest: i.e., not just native, but deglycosylated, active-site 'capped', and other forms of the protein of interest, to enable a careful functional mapping of all surface modifications. Pursuant to this, the methodology could also be re-applied with digests of the protein of interest, to further isolate the locations of post-translational modifications when used in conjunction with other biochemistry methods.

Multicomponent Molecular Analyzer

Multi-component regulatory systems and their variations, often sources of disease, could be studied directly, as could multi-component enzyme systems, using the NTD approach. Information at the single-molecule level may be uniquely obtainable via nanopore transduction methods and may provide fundamental information regarding kinetic and dynamic characteristics of biomolecular systems critical in biology, medicine, and biotechnology. The design of higher-order interaction moieties, such antibody with cofactors and adjuvants; or DNA with TFs, opens the possibility of exploring drug design in much more complex scenarios. One simple extension of this is when the multiply interacting site is simply designed to have an affinity gain. The nanopore transduction detector can be operated as a population-based binding assayer (this would provide capabilities comparable to some SPR-based instruments). The NTD method might also be used to resolve critical internal dynamics pathways, such that the impact of cofactors (chaperones) might be assessed for certain folding processes.

Molecular capture via antibody, aptamer, or MIP capture-matrix & TERISA

It is possible to couple NTD methods with antibody capture systems, or any specific-binding capture system (e.g., MIP-capture, DNA microarray, or aptamer-based capture systems could be used as well, for example) to report on the presence of the target molecules via indirect observation of transduction molecule signals corresponding to UV cleaved NTD 'substrate' molecules (that are freed from the capture matrix). Commercially produced systems are available with matrices pre-loaded with immobilized Fc-binding antibodies; the secondary antibody can then be introduced, and bound by the Fc-binding Ab's, to establish the desired, immobilized, specific-binding matrix (analogous to sandwich-ELISA). If solution with target molecule is now repeatedly washed across the immunosorbant surface, an immobilized concentration of that target molecule can be obtained. We can now introduce our primary antibody that targets the immobilized antigen ('sandwiching' it). If the primary antibody can be

attached to an NTD Biosensing Biomarker as shown in Fig. 17, where the antibody-DNAhp linkage can be broken upon exposure to UV.

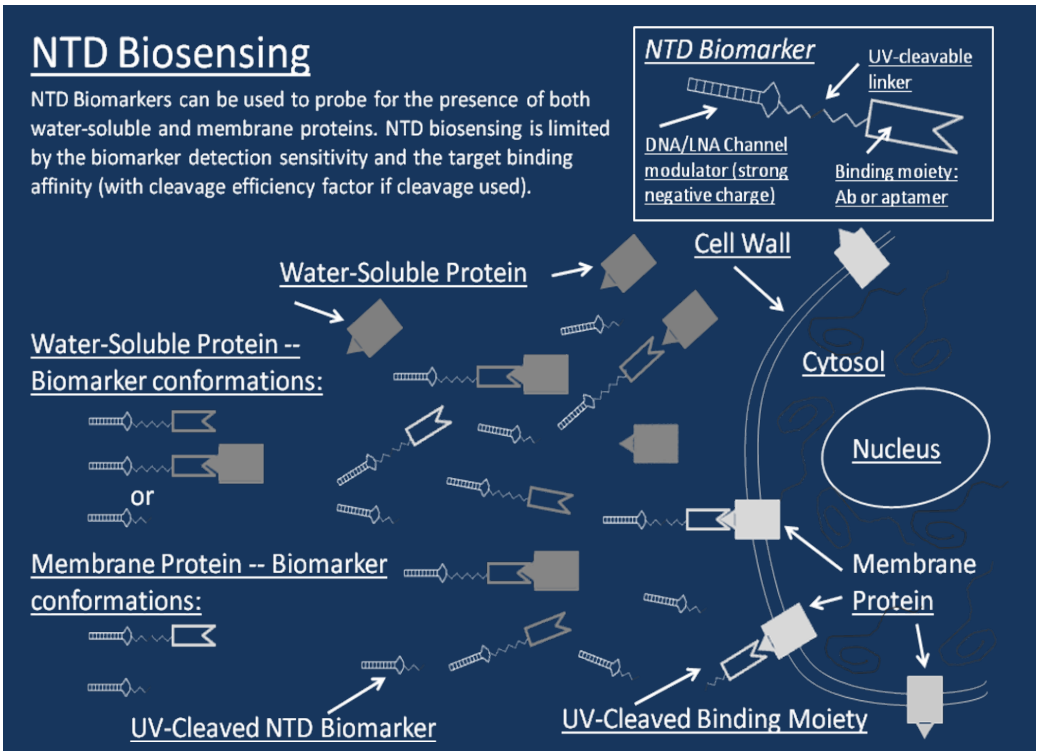

Figure 17. The NTD biosensing approach facilitated by use of immuno-absorbant (or membrane immobilized) assays, such that a novel ELISA/nanopore platform results. The immune-absorbance, followed by a UV-release & nanopore detection process provides a significant boost in sensitivity.

The further novel aspect of this setup is to now have the primary antibody linked to an enzyme that acts on a NTD transducer substrate (analogous to a fluorescent substrate in ELISA). By taking some of the methodology from the ELISA approach (enzyme-linked immunosorbent assay), and merging it with unique aspects of our nanopore detection approach, we have the 'Transducer Enzyme-Release with ImmunoAbsorbent Assay' [92], where "Sandwich TERISA" is assumed to typically be the case, since specific immobilization is desired. This situation is shown in Fig. 18. Also shown in Fig. 18 is an example of an electrophoretic contrast (E-phi contrast) substrate,the idea being to have electro-neutral substrate and upon enzyme cleavage, to leave a highly negatively charged DNA hairpin to be electrophoretically driven ('report') to channel.

Analogous to real-time PCR, where a qualitiative PCR result is self-calibrated according to real-time values to obtain quantitative PCR results, we can do the same with the TERISA and TARISA biosensing methods outlined here. In other words, for all three methods with real-time observation (RT-TARISA, RT-TERISA, E-phi Contrast RT-TERISA), we can shift to a more quantitative footing (as with RT-PCR or RT-ELISA). In our case this is trivially achieved, since the data-acquisition and signal processing is already in use and operating in 'real-time'. This real-time tracking information helps to stabilize the method and complements the biosensing capability with a quantitative assaying capability (where highly accurate resolution of mixtures of DNA hairpin molecules are shown to be possible [5]).

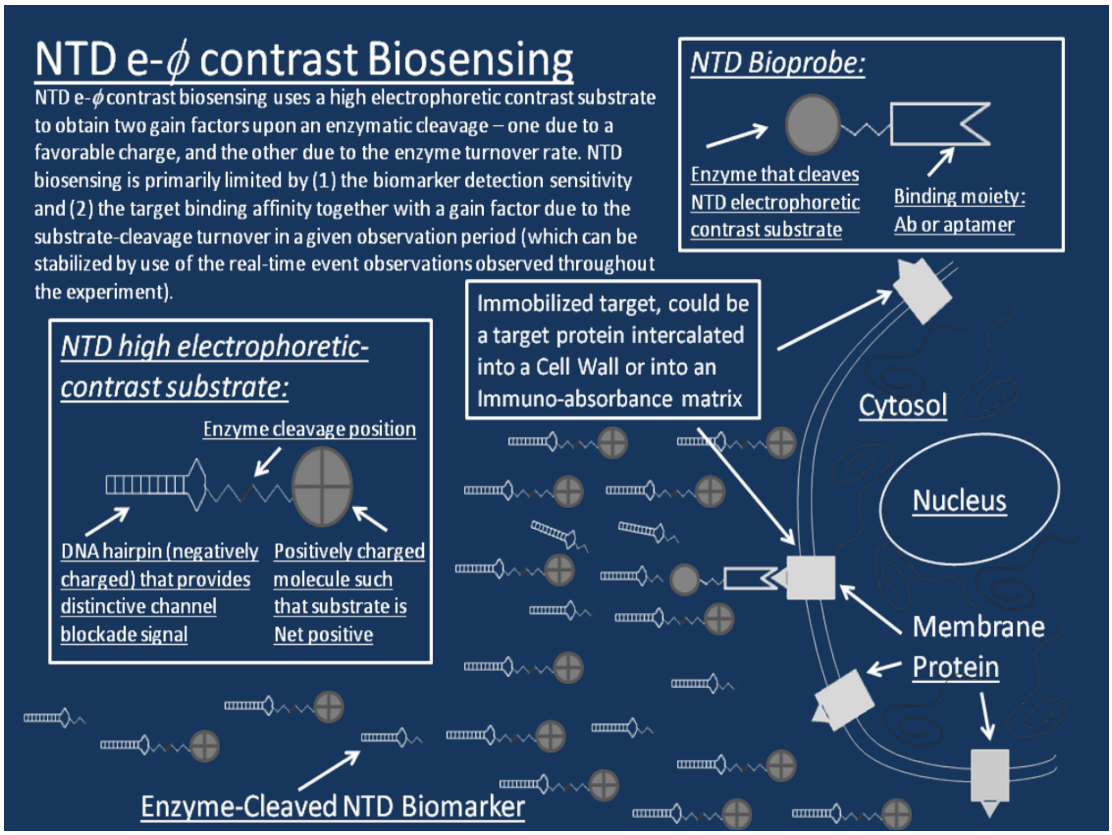

Figure 18. The Detection events involved in the 'indirect' NTD biosensing approaches: TERISA and E-phi Contrast TERISA.

NTD-Gel

Nanopore detectors may offer the separation/identification information of gels, where machine-learning based pattern recognition capabilities, and nanopore-based electrophoresis methods can be used to discern clusters (like the bands or dots in a gel) in a higher dimensional feature space, for greatly improved cluster resolution (such that isomers might be resolvable, etc.). For a nanopore to offer information equivalent to a gel, however, it must also sample a great number of molecules quickly. This requires active sampling control to optimize – i.e., once the sample molecule is identified it is ejected. To this end, pattern recognition informed sampling has been developed and used to boost the sampling rate on a desired species by at least two magnitudes over that obtainable with a passive recording. This lays the foundation for nanopore-based molecular clustering. The separation-based methods still have more information than the separation/grouping of molecules into clusters, however, since they also provide an *order* of separation, according to mobility, or according to isoelectric point, etc. For the nanopore-based methods to recover this critical ordering information on the observed data clusters, something else must be considered. One possibility is the introduction of a mobility reducing agent, such as PEG, into the buffer. The change in average arrival time of the different species after introduction of PEG (using voltage reversal to clear a 'near-zone'), referred to as the 'PEG shift', can then be the basis for an ordering – the least PEG shifted molecules are those, it is hypothesized, with greater mobility and charge (where this is done by comparison of acquisition rates after introduction of PEG and use of voltage control). Just as with gels, all sorts of

functionalized PEG, or other functionalized buffer media, can be introduced for different sieving results, and that provides numerous related functionalizations to the nanopore-gel approach.

Nanopore Processing Unit (NPU)

The NTD foundation with strong ML-based pattern recognition helps to enable an actual chemical computation device, where a fully parallelized, 'chemical' computation can be 'loaded' with choice of buffer and, changes in that buffer, that is sampled with NTD recognition and program/data processing. Akin to efforts in DNA computing, here DNA and DNA synthetics are an excellent material to use in this context, thus the notion, described here, of a nanopore processing unit (NPU). The use of multifunctional NTD transducers (as mentioned above) shows that NPU programming puts long instruction-set coding on the same footing as reduced instruction-set coding (RISC), where the latter has been popular with solid-state CPU's due to their less restricted pipelining (since CPU is not truly parallel as with the 'chemical computing' measured in the NPU). This doubly emphasizes the possible computational-speed benefits of massive parallel computation in properly programmed/utilized NPU component(s) in a standard computer (akin to the common GPU enhancement in vector processing already complementing CPU functionality). The more sensitive TERISA biosensing method, for example, benefits from the off-channel, fully parallelized, 'chemical' computation that is sampled with NTD recognition.

Pathogen Detection

In clinical diagnostics, as well as in biodefense testing, patient blood samples can be drawn for the purpose of assaying the DNA content. Obviously there will be a preponderance of human DNA in such a sample, but if there is infection, then trace amounts of the associated viral or bacterial DNA will be present as well. The question then arises as to how to detect unique elements of bacterial DNA sequence that are singled-out for detection, with very high sensitivity and specificity. This may be possible in the NTD approach, with annealing-based detection along the lines described earlier and in [3,39,87], where ssDNA sequences are targeted for detection of approximate length 22 base sub-sequences. A 22-mer is shown in Fig. 10, 'B'-labeled secondary structure, in the leftmost, linear, ssDNA segment. The Y-shaped secondary structure in Fig. 10 ('B') shows the blueprint for a NTD ssDNA probe for any targeted ssDNA segment, upon 'recognition' (annealing-based), a Y-shaped channel modulator is engineered to occur. If the correct modulator is not annealed, due to a few mis-matches or inserts (particularly at the Y-nexus), then the difference can be discerned with high discrimination. All that is needed is a specific set of enzyme digestion steps on the DNA sample to 'chop' it into shorter segments, and leave targeted regions at the ends of (some) of the resulting ssDNA digests → so as to obtain-dsDNA annealed targets with probe match as in Fig. 10('B'), where the excess ssDNA length (beyond the 22-mer match template) is left to dangle off of one end, as shown for the eight-base segment shown in Fig. 10('B'). In ongoing work, target-segment annealing with high specificity is being explored in the presence of large polymer extensions to the annealing target.

Prototype Stationary statistics based DNA sequencing approach

Nanopore transduction detection (NTD) measures the single-channel current blockades that result from different molecules, *and their different complexation states (and state transitions) involving external target molecules*, where their blockade signals are classified using machine-learning methods. The alpha-hemolysin based NTD biosensor is shown in a close-up of a NTD

channel with modulator present in Fig. 19, where the modulator is covalently bound to a processive DNA enzyme (lambda exonuclease).

It may be possible to discriminate between the four nucleotides that are excised by lambda exonuclease as it enzymatically and processively excises the 3' strand of bound duplex DNA. Other exonucleases are of interest as well, but lambda exonuclease is known to work in a broad range of buffer conditions, including the standard buffer conditions used in the nanopore detector (with magnesium present as co-factor). It may be possible to sequence the DNA by observing the different back-reaction events (probably conformational-change mediated) that are observed with an enzyme-coupled NTD modulator according to whether an 'a', 'c', 'g', or 't' is excised. If the NTD modulator is engineered as desired, it will be possible to enable a useful coincidence detection event via the associated translocation disturbance associated with the excised nucleotide (described in Methods).

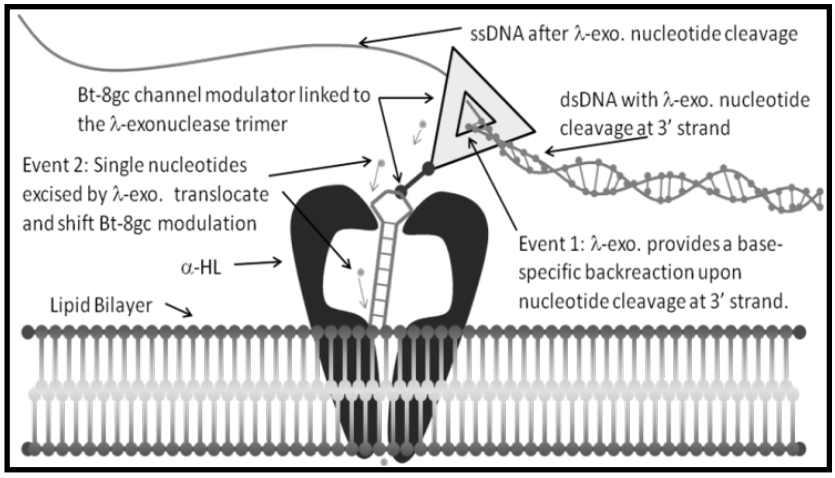

Figure 19. Schematic diagram of the nanopore transduction detector. A Bt-8gc DNA hairpin captured in the channel's *cis*-vestibule, with lambda nuclease linked to the Bt-8gc modulator molecule as it enzymatically processes the duplex DNA molecule shown.

Fundamentally, the weaknesses of the standard ensemble-based binding analysis methods are directly addressed with this single-molecule approach. The role of conformational change during binding, in particular, could potentially be directly explored in this setting. The overall *cheminformatics-integrated* approach also offers advantages over other nanopore detection approaches in that the transduction mechanism that is enabled can now be used to provide two strong mechanisms for boosting sensitivity on single-molecule observation: (i) engineered sensitivity in the transduction molecule itself; and (ii) machine learning based signal stabilization with highly sensitive state resolution. NTD used in conjunction with recently developed pattern recognition informed (PRI) sampling capabilities greatly extends the practical, rapid-assay, usage of the single-channel apparatus and lays the foundation for nanomanipulation (where we have a single-molecule under active control in an adaptive environment). For medicine and biology, NTD methods may aid in understanding multi-component interactions and aid in designing co-factors according to their ability to result in desired binding or modified state.

Alternative prototype stationary statistics based DNA sequencing approach

In Fig. 20, is shown a close-up of a nanopore detector channel with segment of dsDNA captured at one end. It may be possible to sequence DNA by using pattern recognition informed sampling on 'Sanger mixtures' obtained in the Sanger sequencing protocols, where electrophoresis is not used to separate the molecules according to length (although this may be still employed to enhance length discrimination if necessary). At this stage the length 'separation' is done on a single-molecule pattern-recognition basis, simultaneous with reading the end of the dsDNA molecule. The terminus read-out and length evaluation is obtained from channel current blockade observations during capture of the molecule (Fig. 20). The terminus identification is thought to already be possible from preleiminary studies. Length discrimination may be possible via the same modulatory approach used in terminus identification. The key aspect of the success of the length discrimination method lies in the fact that the physical mechanism (producing the discriminatory signal found to be useful) need not be understood. Rather, a model-independent machine-learning approach to the signal analysis can latch onto discriminatory aspects of the information. SVM are used for that purpose here, with feature extraction performed by a HMM.

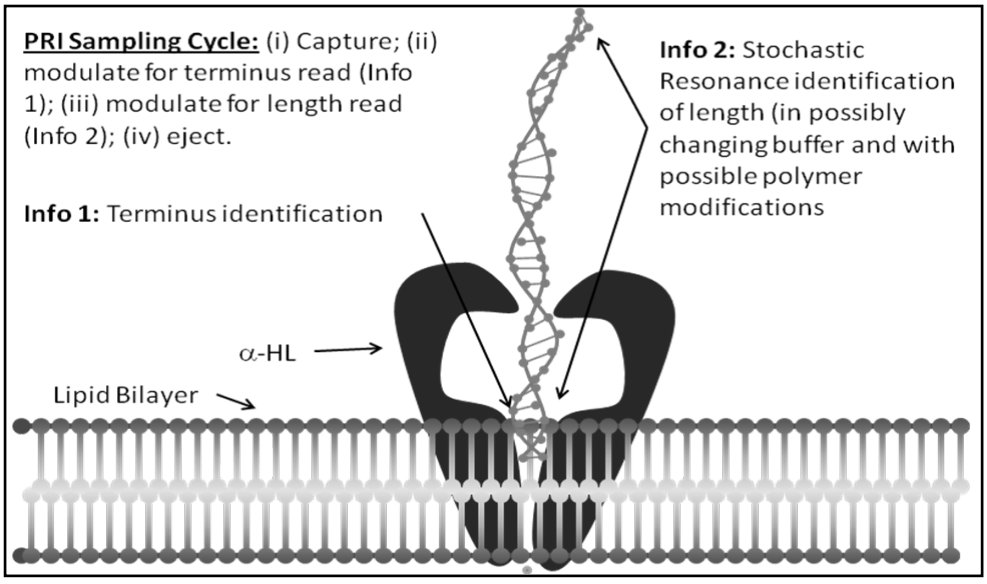

Figure 20. A blunt-ended dsDNA molecule captured in the channel's *cis*-vestibule.

Conclusion

Preliminary tests indicate that it is possible to directly detect annealed vs non-annealed, bound vs unbound, and cleaved vs uncleaved, NTD-probes. This, and other work presented, provides an extensive set of Proof-of-Concept results for NTD-based biosensing and assaying. NTD methods offer improved SNP detection, among other things, and improved detection of other localized DNA regions in general, such as for application to pathogen detection and forensics, with a profound impact on how pathogen and SNP detections are done in diagnostics. NTD methods are shown to offer significant advantage in the aptamer and antibody based biosensing systems as well. Currently there are a huge variety of tests for various forms of cancer, or genetic disorder, for example, and the NTD approach offers a highly-accurate, inexpensive, and fast, test result on

a platform functionalized for all tests – where the tests may also be done simultaneously on the same sample by using a mixture of the appropriate NTD probes.

Nanopore transduction detection provides a highly discriminative method for biosensing, assaying, and performing medical diagnostics using highly specific binding to some biomarker (e.g., antibody-based binding or aptamer-based binding). Kidney damage, for example, is often undiagnosed until significant kidney damage has already occurred and preventive action is limited [86,95-97]. It is hypothesized that NTD probes can also be developed for early stage kidney disease detection, and other disease detection, via biomarker biosensing with femtomolar to attomolar sensitivity, without loss of necessary specificity, in the standard clinical tests of the future. The overall market impacted by this technology is very broad and includes the diagnostics, pharmaceutical, and biotechnology industries.

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Markov models and Support Vector Machine signal boosting; and/or (iii) topological structure identification; and/or (iv) multi-track, parallel, or holographic HMMs; and/or (v) distributed HMM methods via Viterbi-path based reconstruction and verification; and/or (vi) adaptive null-state binning for O(TN) computation. Patented, February 2010.
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