1. FINAL PUBLISHABLE SUMMARY REPORT

As the costs of DNA sequencing continues to drop, the complete genome sequences from thousands of pathogens are becoming broadly accessible. The utilization of this new knowledge in clinical practice, however, critically depends on the availability of new tools and techniques that can quickly and efficiently detect the presence of specific genomic variants of pathogens or human cells. Current approaches rely on costly and error prone amplification methods to achieve the specificity and quantity required by standard means of detection. To address this need we develop a low-cost, single-molecule, DNA barcoding (or genotyping) method, which relies on *solid-state nanopore* based sensing of individual sequence-specific markers.

A solid-state nanopore is a synthetic device composed of a nanometer-scale pore fabricated in an ultra-thin membrane. Nanopores utilize a simple electrical sensing principle: when biopolymers such as DNA or RNA are electrophoretically threaded across a pore, the ion current flowing through the pore is partially blocked, thus providing a mean to sense individual molecules. It has recently been demonstrated that solid-state nanopores may be used to detect sub-molecular structural alterations in both DNA and RNA, as well as proteins. Another practical feature of solid-state nanopores is their ability to actively funnel and subsequently capture extremely small copy numbers of DNA molecules. Today nanopores are considered to be a promising platform for molecular diagnostics, particularly for those involving limited sample quantities. To date however, solid-state nanopores have seldom been utilized for molecular diagnostic sensing applications, primarily due to their inherent lack of DNA or RNA sequence specificity.

In order to achieve sequence-specific sensing of single DNA molecules we developed *two complimentary nanopore-based methods*. In *Phase I* of *Genotyping Nanopores* we utilized Peptide Nucleic Acid (PNA) molecules, which are known to exhibit an extremely high affinity for double-stranded DNA and can invade duplexes with high specificity. We hypothesized that the compact PNA/DNA triplex structure would form a clear signal spike when passing through the nanopore. Our results confirmed this hypothesis showing that PNA/DNA triplexes generate clearly resolved electrical current blockade spikes (Figure 1), thus enabling us to "barcode" specific DNA sequences. The first Aim of this Phase involved optimization of the nanopore size to allow sensing of individual PNA along the target double-stranded DNA (dsDNA). Typical results are shown in Figure 1: the ion-current signal corresponding to the passage of a dsDNA molecule on which three PNA molecules were bound displays three distinct features thus permitting straightforward identification of the DNA molecules as they translocate through the pore. Furthermore, we have demonstrated the feasibility of the *Nanopore Genotyping* method, by performing a real-time identification and classification two nearly identical genes from two human immunodeficiency virus (HIV) subtypes (>92% similarity), which were originally extracted from unmarked human samples. Three PNA molecules were used to uniquely tag either gene. The nanopore classification method permitted a rapid and

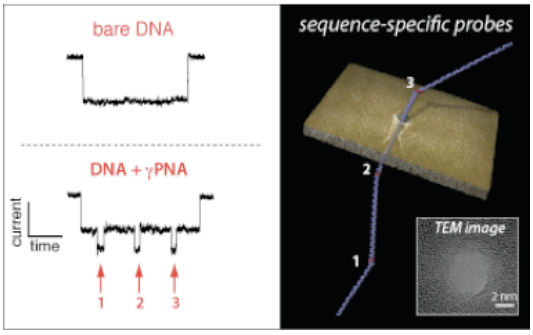

Figure 1 *Nanopore sensing of PNA/DNA complexes.* PNA molecules are used to create sequence-specific markers along DNA, and are sensed by threading the molecule through a ~4 nm solid-state pore (inset: an electron microscopy image of the pore).

highly accurate discrimination and quantification of the two HIV variants. These results were published: Singer A. et al. *Nano Letters*, **12**, 1722-28, 2012.

In *Phase II* of the project we extended our approach and utilized light-emitting "molecular beacons" in order to color-code sequence-specific probes, thus creating a more general method for DNA barcoding, which is also compatible with single-stranded DNA (ssDNA) and ssRNA molecules. In this method, the probes are hybridized to a reporter ssDNA molecule, which consists of a sequence-specific portion matching the target gene of interest, and a barcode region matching a specific set of the molecular beacons. A 3.5 nm solid-state nanopores are used to electrophoretically unzip the molecular beacons hybridized to the reporter strand as illustrated schematically in Figure 2, left. When the reporter strand is threaded in the pore, it slides down until the first beacon encounters the pore surface. Since the nanopore diameter is too small to allow duplex DNA to go through it, a shear force is applied on the beacon. Upon unzipping, the fluorophore of the next beacon is un-quenched and begin to emit photons, which are collected by our sensitive microscope. The unzipping events are probed as a series of multi-color photon bursts, as can be see seen in the red/green photon traces. In this example, 5 units barcode (G-R-G-G-R) was read. These results were recently published: Assad O. et al. *Nano Letters*, **15**, 745-752, 2015.

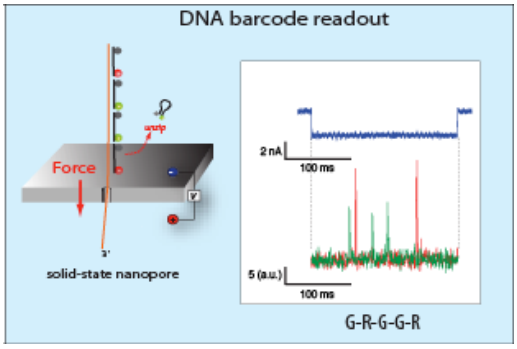

Figure 2 *Opto-electrical DNA barcoding using solid-state nanopores.* Color-coded molecular beacons (shown in Green and Red) are hybridized to a reporter DNA strand to create sequence-specific unique barcode. Unzipping of the molecular beacons produce discrete photon bursts which is read by the opto-electrical nanopore system.

Potential impact and use of our studies

With the completion of project, we demonstrated for the first time the ability of solid-state nanopores to barcode, or *genotype*, individual DNA molecules. Previous to this study solid-state nanopores have proven to be one of the most sensitive and effective single-molecule biosensors, permitting the detection of as low as a few atto-moles (10^{-18} moles) of DNA copies. However, sensing with solid state nanopores lacked the sequence specificity required for most biomedical applications. ***Genotyping Nanopores'*** *main aim was focused on addressing this fundamental deficiency*. With its completion, this project opens up multiple possible directions for real-life applications in biomedical research bearing significant societal impact. Specifically, the barcoding methods can be used for the detection and classification of mutations in oncogenes and related genes for early detection of cancer in circulating tumor DNA. Another application involves an amplification-free and ultra fast identification of antimicrobial resistance pathogens using nanopore barcoding.

These projects have already been launched in the PI's lab, and if successful would have significant impact on society and biomedical care. More information about this project as well as others can be found on our group web site:

http://meller.bm.technion.ac.il/Research_Nanopore_main.html