

Development of Evaporating Droplet Array for Universal Nucleic Acid Quantification

Scope and Goals

In recent years, advancements in molecular biology, particularly in the areas of nucleic acid biomarker discovery and epigenetic research, have opened up a wide array of potential biomedical applications. With new opportunities found in miRNA diagnosis [1-3], pathogen DNA identification [4-6], and early stage circulating cancer marker detection [7,8], novel measurement technologies have become necessary to fully implement these exciting applications and to transform the basic scientific discoveries into commercial products critical to early disease diagnosis and detection.

The capability to accurately quantify low abundance, sequence-specific nucleic acid fragments in a portable, speedy and cost-effective manner is the ultimate goal of both research and industrial communities. Currently, the detection and quantification of nucleic acids is limited to a few options, primarily based on the polymerase chain reaction (PCR) and the sequencing platforms. While both technologies have been widely adopted in the past two decades, fundamental disadvantages, including the necessity to enrich low abundance samples, amplification bias (i.e. low GC vs high CG content fragments), and the requirement of costly, temperature-sensitive amplification reagents currently prevent them from being ideal solutions for applications beyond the laboratory environment.

In our current proposal, the collaborative team wishes to present an ultra-sensitive, on-chip sample concentration-detection device for nucleic acid (in the form of DNA or RNA) detection and quantification. The team's proprietary platform [9] can enrich and concentrate nucleic acid samples by over 10,000 times to allow low-abundance detection without biochemical amplification. Specifically, the proposed technology does not require the thermal cycling component, costly enzymes, or sequence specific labeling procedure, and is a sensitive, cost-effective and simple DNA/RNA measurement solution. ***The completion of the proposed research will provide direct contributions to point-of-care disease diagnosis, real-time pathogen detection, and early-stage cancer detection applications.*** Furthermore, the versatile nature of this platform technology will create immediate commercialization and tech-transfer opportunities for multiple industrial sectors.

Table 1. Comparison with Current Technologies

Technology	Manufacturer	Processing time (post extraction)	Detection limit	Sample volume	Amplification	Instrument cost	Consumable and Reagent cost
qPCR	Life Tech., BioRad, Perkin Elmer	2hr +	pM	10-100 uL/reaction	Required	\$20K	\$100-\$200/96 well plate
Digital PCR	Formulatrix, Life Tech	2hr+	pM	10-100 uL/reaction	Required	\$100K	\$100-\$200/96 well plate
Sequencing	Illumina, Roche 454, Life Tech	2-7days	pM	10-50 ul/reaction	Required	\$50K-\$700K	\$5,000 per flowcell
Proposed device	UCSD/Lamar Team	30 min	10fM	10-50 μ L total	Not required	<\$5K	<\$50

As a platform technology intended for quantitative assay and in vitro diagnostic application, the device needs to accurately, reliably and cost effectively perform the analysis of low copy number circulating nucleic acids in

clinical, point of care, and first-responder settings. This approach will focus on addressing the following challenges:

Accuracy: Because the levels of biomolecular markers such as circulating microRNAs (miRNAs) are generally scarce (as low as 10 fMs) in biological samples, the reading not only needs to have a quantitative accuracy within 15% of its actual level, but also must be highly replicable. Most test methods produce much larger than required uncertainties of nucleic acid levels. It is worth pointing out that, even at 10 fM concentration, 10 μ L of blood contains over 50,000 copies of nucleic acid molecules; therefore the rareness of DNA or RNA is **not** a major source of error. The major sources of error are usually (a) variations in nucleic acid collection efficiency, (b) variations in binding efficiency and specificity of hybridization to probes, (c) noise from the readout due to low signal levels, (d) degradation of nucleic acids over time, and (e) sample handling and contaminations.

Reliability: Essentially all DNA/RNA quantification technologies today require enzymatic amplification process to enrich samples. It is worth noting this step often creates sequence related bias due to an unequal amplification of CG vs. AT regions of nucleic acids. Moreover, dependence on the degradable enzymatic reagents and thermal cycling components inevitably introduce complexity and reliability issues to the detection system. The fundamental solution to these issues is to utilize a different enrichment strategy that does not involve polymerase enzyme and temperature changing modules.

Cost: Most current methods extract, concentrate, and/or purify miRNAs from biological samples for quantitative RT-PCR or sequencing assays. The cost of reagents (polymerase enzyme, SYBR/Taqman dye, PCR plates, probes, dNTP...etc) adds up to over \$100 per test for PCR and an order of \$3000 for sequencing when consumable flowcells are required, which is cost inhibitive for point of care and many clinical applications. The most promising approach for point-of-care diagnosis is on-chip detection without quantitative enzymatic amplification, providing that the issues of accuracy and sensitivity can be properly addressed.

Besides accuracy, reliability and cost, the time from sample to results, the complexity of operation, and the size and portability of the instrument are all additional concerns. In this DoC/MML/NIST project, the team propose ***a lab-on-a-chip platform technology for nucleic acid detection and quantification in order to address the most critical accuracy and cost issues while simultaneously offering additional benefits in the operability and compactness for disease diagnosis and pathogen detection purpose.***

Methods

In order to achieve the high detection sensitivity (femto molar range) and accuracy (low false positive and false negative) required for the intended applications, innovations in the following three areas for the platform are made:

1. **Enriching DNA without amplification:** On-chip nucleic acid enrichment using **oil encapsulated nanodroplets** and **nano hybridization reactors**,
2. **Increasing hybridization efficiency:** Implementation of engineered component to introduce *Marangoni flow* to manipulate local nucleic acid concentration and enhance probe-target interaction,
3. **Developing physical models to optimize and predict device performance:** Development of an accurate model and simulation platform as the physical foundation and guideline to optimize the hybridization-detection efficiency.

1. **Enriching DNA without amplification:** On-chip nucleic acid enrichment by oil encapsulated nanodroplets and nano hybridization reactors

To achieve the goal of accurately detecting and quantifying the sequence specific nucleic acid targets in low concentrations, it is essential to enrich them from the original sample sources. Instead of relying on complex chemical methods such as PCR or cluster amplifications (utilized by most next-generation sequencing devices), the core design of our platform is based on the concept of evaporating droplet arrays and nano-hybridizing reactors. The process flow for the sample enrichment and detection scheme are illustrated in Fig. 1.

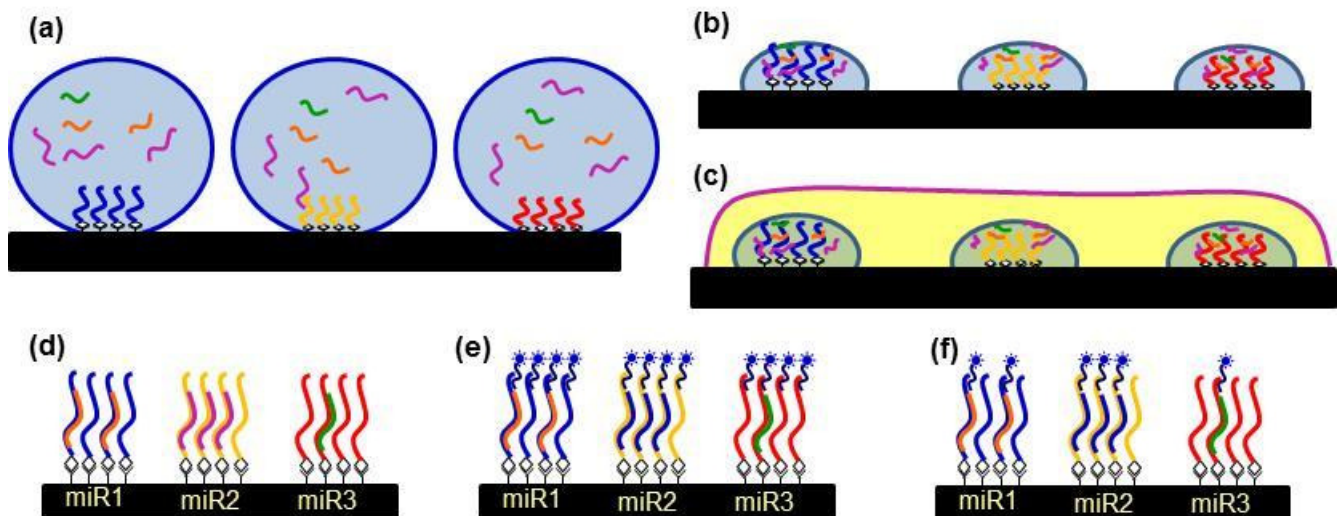


Figure 1: The schematics of nucleic acid detection. (a) Dispensing droplets of samples containing nucleic acid targets (e.g. miRNAs) upon hydrophilic islands with specific DNA oligo probes. The amine end-linked DNA oligo probes are immobilized to aldehyde-activated microislands. The micro-islands are surrounded with super-hydrophobic surface formed by black silicon. (b) The concentration of the target nucleotides is increased as the volume of the sample droplet is reduced by evaporation. (c) A layer of oil is dispensed to encapsulate the thin layer of nucleic acid enriched liquid to form highly efficient nano hybridization chambers. (d) Target DNAs are hybridized with the specific probe on each island. (e) Attaching quantum-dot linked reporter oligo to all the probes. (f) Use the pi-stacking effect to selectively remove reporter oligos from probes without hybridization by washing[10-12]. By detecting the Q-dot fluorescent intensity, each nucleic acid marker can now be detected and quantified.

The device consists of a super-hydrophobic surface with hydrophilic islands formed using the **black silicon process** [13,14]. The black silicon has a self-formed nanostructure under plasma etch with SF_6 and CF_4 . Advantages of the black silica surface include an easy manufacturing process, stable structure with long shelf life, and compatibility with a wide range of nucleic acid solvents utilized by common commercial DNA/RNA extraction kits.

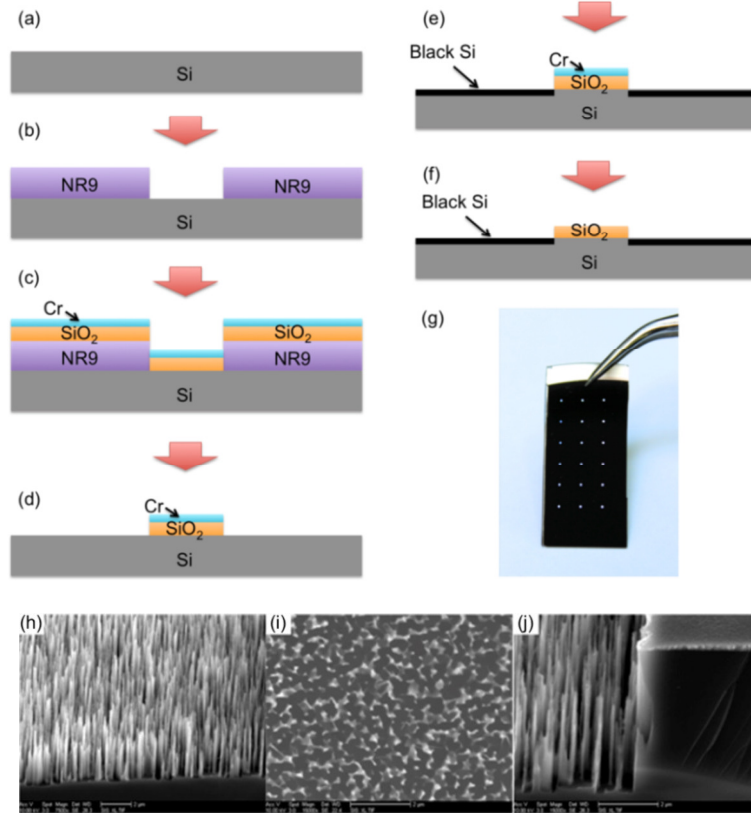


Figure 2: The fabrication process of the microarray for evaporating droplets; (a) mechanical-grade silicon wafer is cleaned for microfabrication; (b) photoresist NR9-1500 is patterned on the silicon wafer by lithography; (c) SiO_2 and Cr layers are deposited on the substrate by sputtering; (d) a microarray of SiO_2/Cr dots is patterned on the silicon wafer; (e) nanopillars, which give rise to the “black appearance” and “super hydrophobicity” of silicon, are etched by the DRIE process; (f) the Cr layer is removed by chromium etchant; (g) a photograph showing the reflectivity difference between black silicon and SiO_2 covered hydrophilic islands (tiny white dots on the picture). SEM images of nanopillars fabricated using the DRIE method: (h) 45° degree view (i) top view (j) interface between the hydrophilic island and the nanopillars. All the scale bars in (h,i,j) are for 1 micrometer.

The workflow of the enrichment process is simple and straightforward. All nucleic acids from various forms of patient samples (serum, urine, swab, etc.) are extracted by using the standard nucleic acid extraction kits. The eluted DNAs are then collected in salt-diluted hybridization buffer. The dilution of salt concentration will provide a favorable DNA hybridization condition in oil-encapsulated nano hybridization chambers where the salt concentration becomes comparable to the normal concentration after droplet evaporation.

The low-salt buffer containing the nucleic acid targets are dispensed onto the device as an array of droplets with an initial droplet volume of 4 μL each. By evaporation, the volume of droplets shrinks and is centered towards the hydrophilic islands without the coffee ring effect, driven by the surface energy [15]. When the droplet volume is reduced to 400pL (concentration enriched by 10,000 times) or a height of the droplet reaches $40 \pm 5 \mu\text{m}$, a layer of oil is dispensed to encapsulate the droplets to form an array of nano hybridization chambers, within which the target nucleic molecules hybridize with the DNA probes immobilized to the

hydrophilic surface [16,17] (see Fig. 3). The height of the droplets is monitored in real time by the focal point of the optic system. Experimentation has shown that a precision of $\pm 5\mu\text{m}$ in the droplet height can be well controlled, and the final test results are not sensitive to the droplet height variations to give the proposed DNA detection method a relatively large tolerance to any process variations and device non-uniformity.

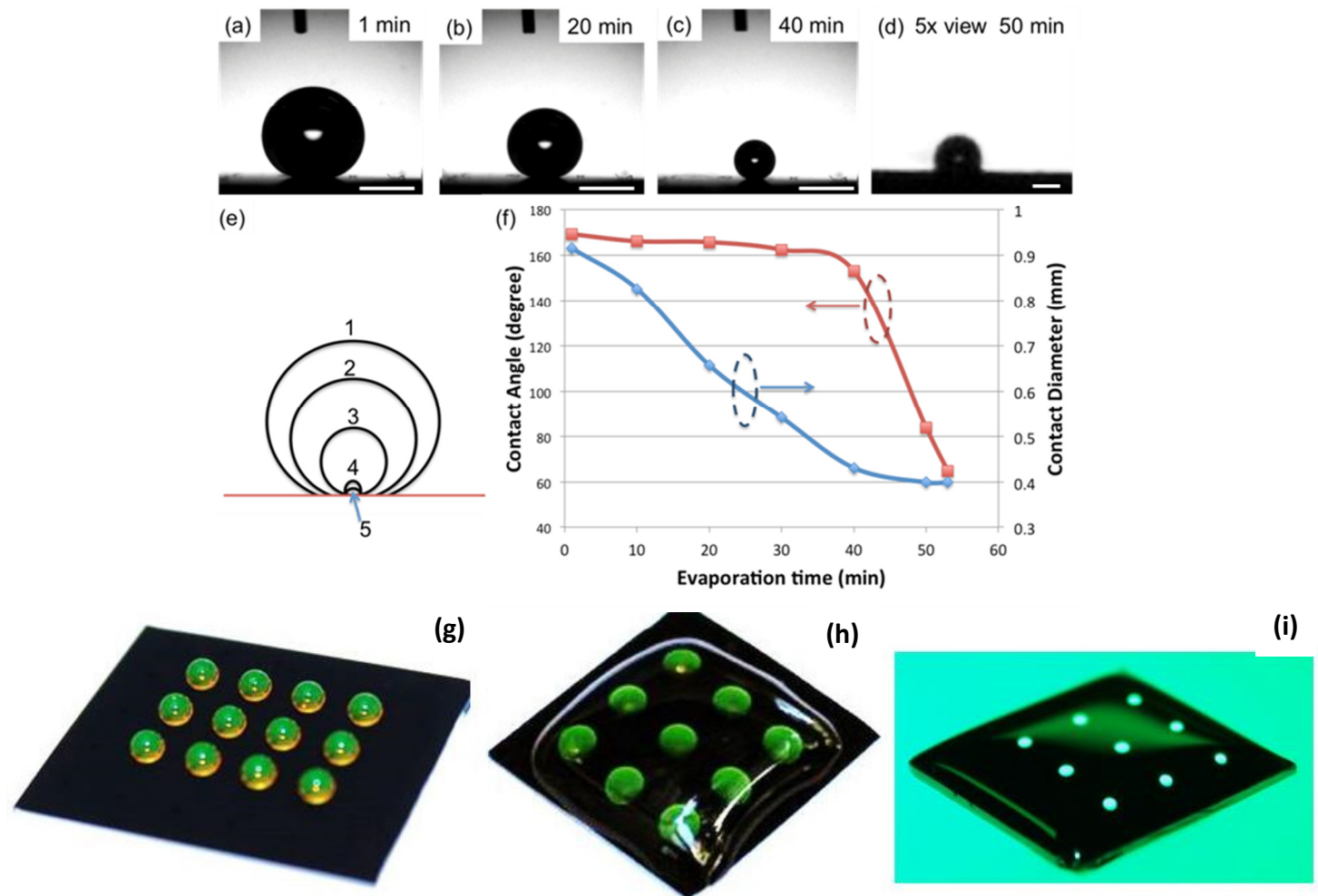


Figure 3: The evolution of an evaporating water droplet on SiO_2 patterned black silicon. (a,b,c,d) photographs of droplet at evaporation time of 1 min, 20mins, 40 mins, and 53 mins. The scale bar is 1mm for (a,b,c) and 200 μm for (d). If desired, the evaporation process can be shortened to < 10 minutes by raising the temperature to 60C. (e,f): schematic and data for contact angle and droplet shape change during evaporation. (g-i): Photographs of droplets containing fluorescently labelled DNA. (g): Droplets as dispensed on black Si; (h): Oil encapsulation when the droplet volume is reduced to 50% of the original volume; and (i): Oil encapsulation when the droplet reaches the desired height (40 μm) and volume. (g) and (h) are photos under unfiltered white light, and (i) is the photo of filtered fluorescence.

2. Increasing hybridization efficiency: Implementation of engineered components to introduce Marangoni flow to manipulate local nucleic acid concentration and enhance probe-target interaction.

After the enrichment step, the team will further incorporate a microfluidic environment to facilitate nucleic acid hybridization and self-align the sample droplet with the position of the detection probe. The driving force of suspended DNA/RNA is a Marangoni flow that can be introduced to the system by different designs such as surfactants [18,19], electrowetting, and asymmetric boundaries of different surface energies. The team

proposes to take the approach of asymmetric boundaries to induce the Marangoni flow that produces a liquid flow from the hydrophobic surface to the hydrophilic surface, as illustrated in Fig. 4.



Figure 4: Creation of the Marangoni flow using two asymmetric boundaries of different surface energies (e.g. hydrophobic and hydrophilic surfaces).

A hydrophobic surface will be placed on the top of the patterned surface with the DNA probes. The Marangoni flow forces the suspended DNA/RNA towards the surface on which the DNA/RN will hybridize with the probes. This phenomenon was confirmed experimentally after the droplet dries completely.

The team has demonstrated the capability to manipulate the hybridization droplet with this technique in Figure 5. Further optimization of functionalized surfaces using SEM/XRD instrument and atomistic molecular dynamics simulations (MD) will not only help to create a radial flow pattern to direct the evaporation droplet but will also enhance the hybridization efficiency. Figure 5 shows continuous motion of a drop shrinking and "swimming" on a solid due to a Marangoni flow that is induced by surfactant gradient.

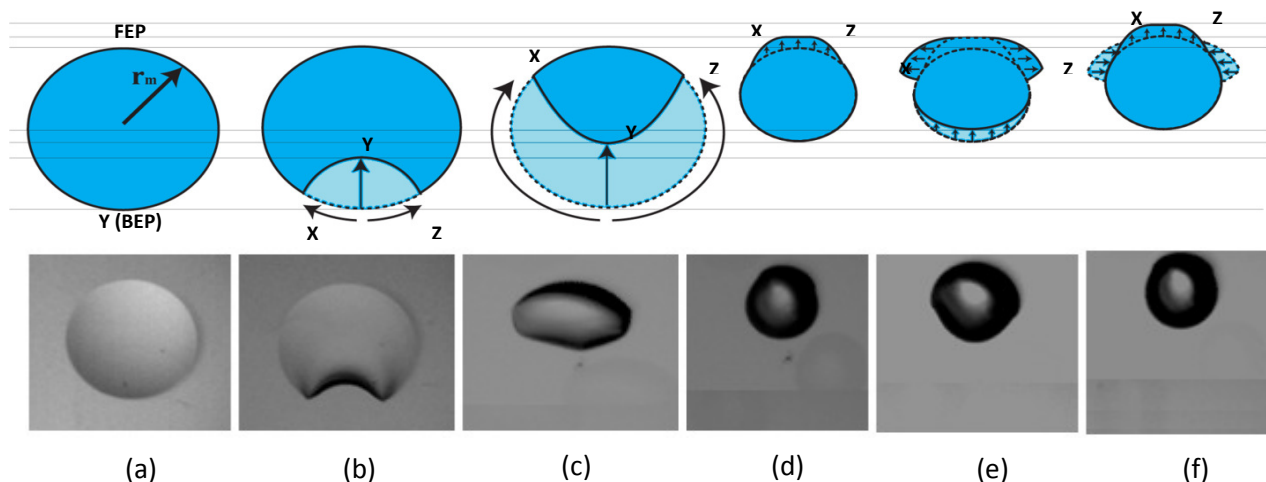


Figure 5: An example of a liquid drop contracted and directed to a designated location via a Marangoni flow. Upper panel: schematic representation; lower panel, frames from a movie. (a) the maximum area or maximum radius (r_m) the drop obtained after placement on the solid; (b) and (c) are the start and end of primary retraction respectively; (d), (e) and (f) the swimming motion following the primary retraction. Time between frames ~ 0.1 seconds.

To demonstrate the effectiveness of the surface treatment, a 4 μL droplet of water with diluted Rhodamine was pipetted onto the patterned black silicon template. Because of the SiO_2 hydrophilic islands, the droplet found a stable area to reside when being dispensed. Due to the large size mismatch between the droplet and the SiO_2 island, the droplet was often initially misaligned with the SiO_2 island. However, as the evaporation process went on, the droplet shrank towards the center of the SiO_2 island until the contour of the droplet was aligned with the boundary of the SiO_2 island (Figure 6). By self-alignment, sample droplets can be easily controlled on the black silicon template, which greatly facilitates the droplet dispensing process for hybridization-detection purpose.

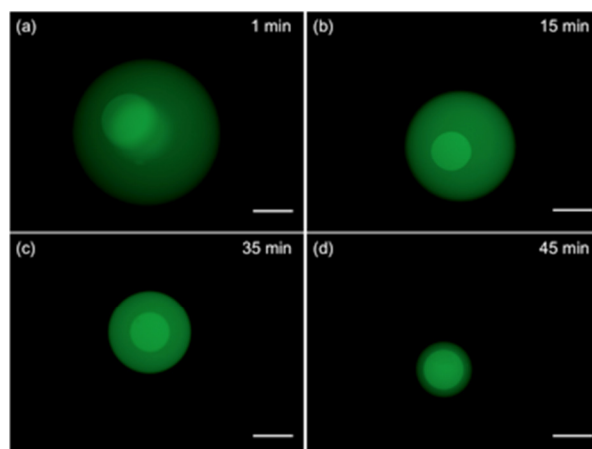


Figure 6: The sample droplet was self-aligned with the SiO_2 island during evaporation: (a) 1 minute; (b) 15 minutes; (c) 35 minutes; (d) 45 minutes. The scale bars are $400\ \mu\text{m}$.

After the evaporation and hybridization processes, the detection sensitivity of the evaporating droplet microarray was tested by varying the target molecule (nucleic acid and protein) concentration from $10\ \text{fM}$ to $100\ \text{pM}$. The total number of bonded QDs was calculated by using a custom Matlab program. As a control sample, one device area had hydrophilic islands pre-anchored with the scrambled probes, so that any quantum dots left in those areas were due to incomplete washing or non-specific binding. The team obtained the real binding events by subtracting the number of non-specifically bonded Q-dots from the detected events over the same area. The results are shown in Figure 7. A linear relationship between the target DNA concentration and the bonded QDs was obtained with the molecular concentration ranging from $10\ \text{fM}$ to $10\ \text{pM}$. For higher target concentration beyond $10\ \text{pM}$, the bonding events were too dense to be resolved microscopically by the image processing program. For concentration lower than $10\ \text{fM}$, the results were less reliable since the number of non-specific bindings became comparable with the number of specific bindings.

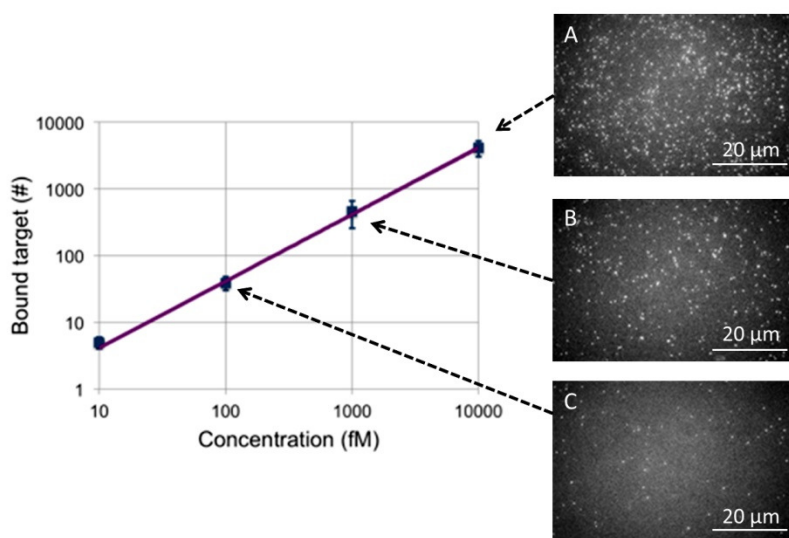


Figure 7: The dependence of the number of hybridized targets and the concentration of target molecule in the sample; (a) the processed image of visualized quantum dots with a target concentration of $10\ \text{pM}$ (a), $1\ \text{pM}$ (b) and $100\ \text{fM}$ (c).

3. Developing physical models to optimize and predict device performance:

In order to facilitate the target-specific hybridization process, it is imperative to maximize the hybridization efficiency between the target molecules and the DNA probes, and to understand the effects of non-specific binding at the molecular level. Empirical and experimental approaches are not sufficient given the large number of parameters that can affect the hybridization process and the complex relations among these parameters. Therefore, the team proposes to develop a physical model, supported by the molecular dynamics (MD) simulation platform, to analytically calculate the hybridization efficiency. The calculated results will be verified by well-planned experiment. The goal of developing such a physical model is to provide a companion tool that will help to optimize various parameters, such as pH, temperature, probe concentrations and ionic compositions, to maximize the hybridization efficiency and detection sensitivity in a scientific rather than try-and-error approach.

The model takes into account the following effects specifically applicable to this device: (a) the transport of target molecules within the nano chamber, (b) the effect of Coulomb interactions that repel the approach of target molecules towards the DNA probes, (c) the field-screening effects of ions in the buffer solution, and (d) the binding kinetics and equilibrium constant of forming DNA/DNA or DNA/RNA duplex [20-26]. A close-form approximate solution from the principles of thermodynamics and transport equations can be found:

$$\eta_t = \frac{1}{\frac{L}{K_{eq} N_s} \left(e^{\frac{\epsilon \xi_T A N_s}{\eta D_T}} + 1 \right)} \left[1 - e^{\frac{-D_T t}{\left(\frac{2L}{\pi} \right)^2}} \right] \quad (1)$$

where η_t : hybridization efficiency, L : height of the droplet, D_T : diffusivity of the target molecule, η : viscosity, N_s : surface density of probes, ϵ : permittivity, ξ_T : zeta potential of the target molecule, and K_{eq} : reaction constant of formation of DNA/DNA or RNA/DNA duplex. Limited by space, the PI does not show the temperature dependence explicitly although several parameters in (1) such as η , D_T and K_{eq} are temperature dependent. Without any fitting parameters, the model has shown excellent agreements with experimental data; some of the results are included in Figure 8.

Figure 8 shows the dependence of calculated hybridization efficiency on the transport properties of the molecules, the equilibrium constant, and the binding specificity. Aided by the physical model, this multidisciplinary collaborative team is able to design and optimize the device structures and operation conditions (e.g. probe density, probe length, operation temperature, ionic strength and pH value, etc.) to obtain high hybridization efficiency and to develop strategies (e.g. on-chip calibration and control area) to cope with non-specific binding.

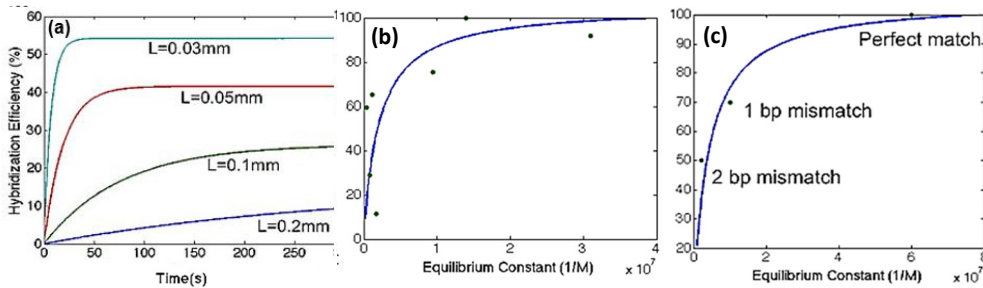


Figure 8: Calculated hybridization efficiency from the physical model and comparisons with the experiment results. The hybridization efficiency is the y-axis for all the figures. All “dots” are experimental data. (a,b,c) show the dependence of the hybridization efficiency on the reaction time of different droplet height, the equilibrium constant of the reaction, and the amount of base-pair mismatch.

Combined with the macroscopic continuum model above, the team intends to incorporate atomistic molecular dynamics considerations to provide insight on the molecular level. In addition, the exact way in which DNA/DNA and DNA/RNA move and approach a designated surface as well as their interactions as functions of the local environment (including biomolecule hydration, ions concentration and surface interactions) can be quantified with the MD simulation.

Atomistic and quantum simulations can probe the molecular events that are difficult to visualize or characterize by experiment, and can also provide insights of DNA/DNA and DNA/RNA hybridization processes that involve multi-scale phenomena. For instance, hybridization process or the droplet evaporation can take many minutes, whereas DNA/DNA and DNA/RNA folding and the surface atoms' structural re-arrangements, which affect hybridization, occur at a much shorter time scale. Consequently, multi-scale simulation approaches are highly desired to achieve a comprehensive, fundamental understanding of the whole chemical and physical processes in order to rationalize experimental design. Combined with the macroscopic continuum model in CFD and the quantum model, atomistic MD simulation can be utilized to correlate the molecular properties and microscopic structure with macroscopic properties, such as hydrophobicity, surface morphology and chemistry of probes into the interfacial phenomena at both microscopic and macroscopic scales, for the purpose of guiding and optimizing the experimental parameters (27).

By grafting peptide mimic SMAs onto the substrate surface, the surface hydrophobicity can be altered. MD simulations would be a powerful tool for experimental surface design. In addition, the exact way in which DNA/DNA and DNA/RNA move and approach a designated surface can be visualized with the MD simulation. These are difficult to understand according to the current experiments and theories, but can be quantified with MD simulation. The molecular properties obtained from atomistic simulation will be used in the macroscopic CFD calculation. This simulation procedure will be utilized to optimize the nucleic acid hybridization parameters for our nucleic acid detection platform. To study the interfacial properties, surface modeling involving establishment of the atomic connectivity and assignment of intra-molecular potentials will be applied. Fig. 9B shows an example system which consists of black silica surface constrained with all atomistic potentials and water nano-droplet.

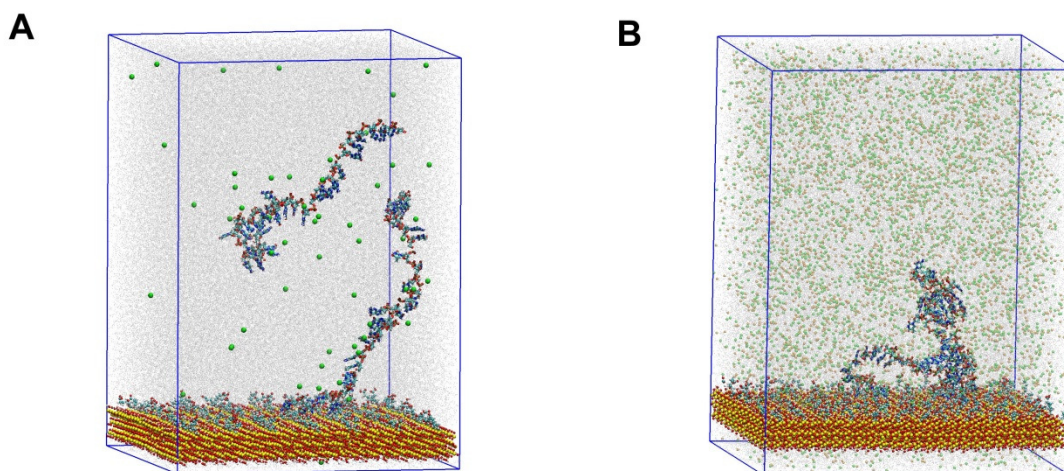


Figure 9: Molecular modeling of target-probe hybridization at buffer conditions at low (A) and high ionic salt (B) conditions during droplet enrichment process. Quantum calculation of Density functional theory (DFT) was utilized to parameterize aldehyde chain and surface silica atoms' partial charges. CHARMM potential was used for atomistic interactions. Substrate surface is represented with α -cristobalite (101) partially hydroxylated and chemically modified with aldehyde chains of density of $0.84/\text{nm}^2$. Colors represent different chemical

components (green: counter ions; red: silicon atoms; yellow: oxygen atoms on silica surface; white: hydrogen atoms; cyan: carbon atom of aldehyde; grey: water)

To enhance the device efficiency and sensitivity, surface morphology and hydrophobicity are the key parameters, which can be controlled by varying chain length, backbone flexibility, and DNA grafting density. The co-PI has more than 10 years of research experience in developing polymer self-assembling monolayer surfaces (SAMs) through both experiments and simulations (28). Using a self-developed surface modeling program, which can efficiently establish the atomistic model for any substrate surface of complicated chemistry and structures, the team can parameterize the system using the methodology of quantum calculations (ab initial and QM/MM) for the proposed MD simulation during the sensor design and droplet evaporation.

With the integration of macroscopic and molecular models, the team will perform multi-scale simulations (MD and CFD) to investigate interactions of nucleic acids as functions of ion concentrations, hydrations, and other chemical factors. In the team's lab-on-a-chip platform experiments, it was found that molecular diffusion of nucleic acids becomes a rate-limiting step for the whole system's efficiency. As the solution droplet evaporates on the surface, the ion concentration increases by several orders of magnitude. Without optimizing the proper ion concentration range, the evaporating phenomena may interfere with the hybridization process. The molecular properties (such as the diffusion coefficient) estimated from atomistic simulations can be incorporated into the macroscopic CFD simulation.

Statement of Work

The proposed project has 3 specific aims: (1) development of the technology platform for high sensitivity, accurate, and low cost detection of nucleic acid targets, (2) development of physical models for rational design of the device and insights for on-chip hybridization process, and (3) verification of the device efficiency, repeatability, accuracy, and utility. The research tasks are described for each aim.

Aim 1: Development of the technology platform for high sensitivity, accurate, and low cost detection of nucleic acid targets

Task 1-1: *Fabrication and test of black Si super-hydrophobic templates with arrays of 150 μm diameter hydrophilic islands immobilized with molecular probes.*

The proposed device consists of an array of hydrophilic SiO_2 islands surrounded by a superhydrophilic surface. The array of hydrophilic islands will be fabricated using the conventional photolithographic method and nanopillars will be formed by deep reactive ion etch (DRIE) over the rest of the Si area to create the black silicon superhydrophobic surface. The hydrophilic islands will be patterned on a pre-cleaned, mechanical grade silicon wafer by negative tone photoresist NR9-1500PY (Futurrex, USA). To create a superhydrophobic surface, nanopillars will be fabricated using the deep reactive ion etching process. Unlike most top-down process for nanostructure formation that requires definition of nanoscaled patterns and pattern transfer, the nanopillars can be formed naturally during the deep reactive etching (Plasmalab System 100, Oxford Instruments) process. This presents a cost-effective method of forming superhydrophobic surfaces over large areas. In the DRIE process, SF_6 gas is flowed at 30sccm during the 8 seconds of reaction time, followed by a passivation cycle when C_4F_8 gas is flowed at 50 sccm for 7 seconds. After 80 etching/passivation cycles, dense arrays of nanopillars can be formed with an average pillar height of 4.5 μm .

To functionalize the SiO_2 hydrophilic surface, the PI will immobilize the DNA probe on the SiO_2 surface. At first, aminopropyl-triethoxysilane (APTS) will be employed to convert surface silanol group (SiOH) to amine group

(NH₂). The silicon atom in the APTES molecule will form a chemical bond with the oxygen of the hydroxyl group (OH). Next, glutaraldehyde (GTA) will be used as a grafting agent for DNA immobilization. GTA binding is achieved through its aldehyde group (COH) by forming a chemical bond with the amino group of APTES. For DNA probe immobilization, DNA oligonucleotides with an amine group at 3' end will be linked to the aldehyde group of the linkers. The target nucleic acids will be hybridized with the DNA probe of complementary sequence.

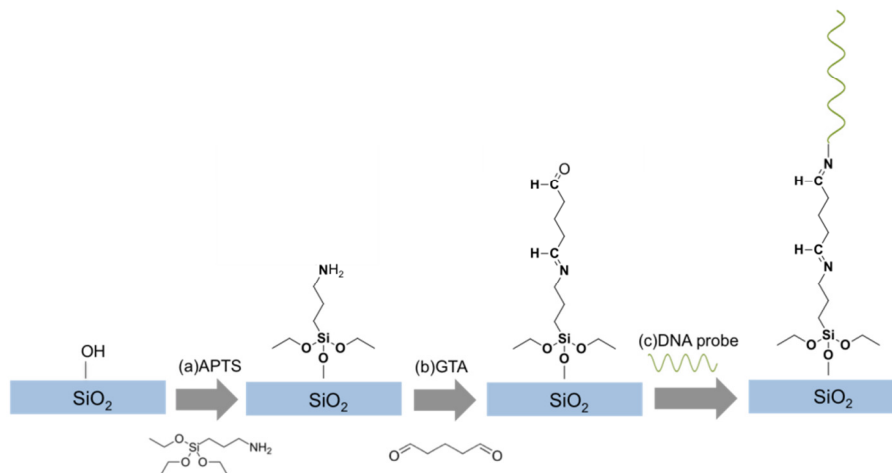


Figure 10: Process flow to anchoring DNA probe to the hydrophilic island: (a) The surface is linked to APT; (b) the aldehyde group of GTA is bonded to the amino group of APTS; (c) the DNA oligonucleotide probe with amine modification at the 3' end is linked to the GTA.

In this proposal, the team plans to produce 30 black silicon wafers with hydrophilic islands functionalized with different DNA probes for technology development and evaluation. These wafers will be used to prove the basic concept of molecular concentration enrichment without amplification, characterize the hybridization efficiency, and optimize the hybridization conditions for different target DNAs.

Task 1-2: Characterization of the device sensitivity and specificity using synthesized DNAs and RNAs.

The device sensitivity can be characterized rather easily by following the same process described in the previous section (e.g. Fig. 7). Several synthesized DNA and RNA probes of different lengths (e.g. 20mers to 200mers) and different C-G content (e.g. from 30% to 70%) will be used to measure the sensitivity and hybridization efficiency. We will also find the optimal experimental conditions, including hybridization temperature, PH value, ionic strength, and probe density, will be found for the highest sensitivity.

A more challenging problem is specificity, an area which will be specifically investigated in this proposal. Two factors could increase the rate of false positive: incomplete wash of Q-dot labels and non-specific DNA hybridization. The effect of incomplete wash of quantum-dots can be minimized by improving the washing process and surface passivation. BSA and 5% non-fat milk which contain BSA and other proteins will be used to eliminate possible sites for non-specific Q-dot binding. A more challenging task is to distinguish non-specific binding due to low (1-2 bp) number of mismatched base pairs. For certain applications such as bacteria detection for infectious disease, such non-specific binding is lesser of a concern. However, to apply the same platform to miRNA detection, non-specific binding can become a main concern since many miRNAs differ in their sequence by only 1 or 2 nucleotides.

Non-specific binding occurs when the energy difference caused by low number of mismatched base pairs is relatively small. For given a DNA, the effect of non-specific binding due to partial base-pair mismatch is highly dependent upon the design of the probes. General guidelines exist for the DNA probes, but to develop an effective approach, a quantitative model will be required to help the probe design.

As shown in Fig. 8(c), the team has developed a physical model that can relate the probability of non-specific binding to different number of mismatched base pairs for a given probe design (including the probe length and the GC content). For example, for a 20 nt DNA probe with 40% G-C contents, the non-specific binding efficiency is about 70% and 50% with single and double base-pair mismatch compared to the efficiency for perfectly matched DNA target. The relative non-specific binding efficiency drops to 5% or <1% when there are 3 or 4 mismatched base pairs. This analysis suggests that the team can design multiple DNA probes (or a probe set) for the DNA target to obtain < 1% false positive. In a probe set, one probe will have the perfect match, and the other three probes will have 1, 2 and 3 base-pair mismatch with the target, thus yielding the mismatch bp numbers (0, 1, 2, 3) for the 4 probes. The ratios of the hybridization efficiencies among these probes will be analyzed. The true binding with the target DNA is expected to show the characteristics of Fig. 8(c). If the binding is caused by non-target DNA, then both the absolute hybridization efficiency and the ratios of the efficiency among the probes will be skewed significantly, signifying the occurrence of significant non-specific binding.

Aim 2: Development of physical models for rational design of the device and insights for the on-chip hybridization process.

Task 2-1: *Development of nucleic acid hybridization model based on the transport equations, reaction constant, and kinetics.*

As explained previously, there are far too many interactive parameters to optimize the process based on empirical or intuitive approaches. It is crucial to guide the development based on a physical model that includes all the first order physical effects. The team will formulate the problem using the transport theory taking into account the diffusion and drift properties of molecules under proper boundary conditions. To simplify the mathematics but retain the key physical phenomenon, the team will formulate the problem in one-dimensional Poisson-Boltzmann equations. Although the problem has been studied extensively, this model will include the following unique characteristics that will generate additional insights closely related to our specific applications:

1. The team will consider the steric hindrance effect and the Coulomb repelling force produced by the anchored DNA probes. Although without the atomistic model, one cannot formulate these effects from the first principle, empirical parameters can be introduced and have their values determined from experimental data. For example, the parameter “A” in Eq. (1) is used as a parameter to measure the strength of steric hindrance and Coulomb repelling effect by the probes. Although its value cannot be calculated, the team can obtain its value by measuring dependence of the hybridization efficiency on the length (number of base pair) of the DNA probes and the surface density of the probes. One such example is shown in Fig. 11.

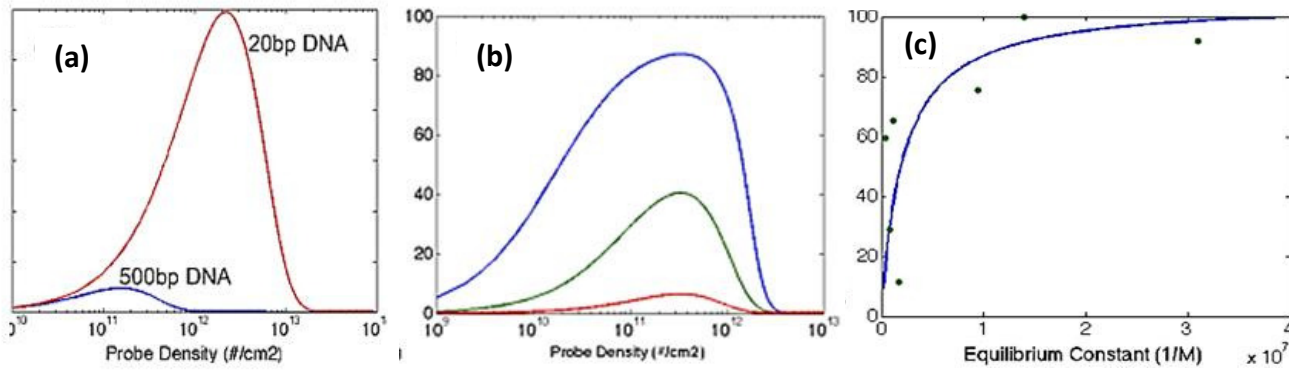


Figure 11: Calculated dependence of DNA hybridization efficiency on (a) length of probe and target molecule, (b) surface density of probes, and (c) reaction equilibrium constant. Dots are experimental data. From these results one can obtain the coefficient “A” in Eq. (1) that measures the strength of the steric hindrance and Coulomb expelling force between the probes and target DNA.

2. The effects of ionic strength, PH value, and hybridization temperature. The model will take into account all the ionic components in the oil-encapsulated nanochambers. The nonlinear Poisson-Boltzmann differential equations can be solved without using the Debye length approximation. This is important since the Debye length that measures the field screening effect can be comparable to the effective length of the single strand DNA probe. Furthermore, the presence of bivalent ions in the buffer can have strong effects on the hybridization.

In addition, the effect of hybridization temperature will be included in the model. Temperature affects three parameters: viscosity, the diffusivity of the molecules, and the equilibrium constant for the hybridization reaction. The hybridization efficiency decreases when the temperature is close to the melting point. On the other hand, low hybridization temperature not only decreases the diffusivity but also degrades the specificity. Therefore, the optimal hybridization temperature depends on the design of the probes, the chamber geometry, the density of probes, and the ionic strength. For example, one important result from the physical model is that to achieve high hybridization efficiency or sensitivity, the necessary condition is: $L < K_{eq}N_s$ (i.e. the liquid thickness of the nanochamber has to be shorter than the product of the reaction equilibrium constant and the surface density of the probe). This criterion not only underlines the great advantages of using nano hybridization chambers having the liquid thickness (20 μm) 100 times smaller than the thickness of 96-wells, but also indicates the importance of temperature effect that determines the magnitude of the reaction constant K_{eq} .

In this task, the team will develop a comprehensive model to guide the technology development and process optimization. Although many of the effects mentioned here have been studied and reported in literature [20-26], one cannot find a comprehensive physical model that is specially designed for the proposed device with all the key effects included. Above all, the model can be experimentally verified and the parameters in the model can be found experimentally.

Task 2-2: Development of atomistic model to understand the molecular dynamics of DNA hybridization on the solid substrate.

Combined with the macroscopic continuum model in computer fluidic dynamics (CFD) and quantum calculations, the team will employ atomistic molecular dynamics (MD) simulation to correlate the molecular properties and microscopic structure with macroscopic properties, such as hydrophobicity, surface morphology

and chemistry to probe into the interfacial phenomena at both microscopic and macroscopic scales, for the purpose of guiding and optimizing the experiments.

In order to simulate the interfacial properties, the team will establish atom connectivity and assignment of intra-molecular potentials. Figure 12 demonstrates a system which consists of a black silica surface constrained with all atomistic potentials and nano-droplet. Using this platform, we will establish a substrate surface model and then parameterize the system using the methodology of quantum calculations (ab initial and QM/MM). Currently, the simulation platform and experimental data have suggested the ranges of detection oligo density (10^7 to 10^{12} oligos/cm²) and salt concentration (>0.5 M NaCl) as the optimal condition to facilitate the hybridization during the droplet evaporating process (Fig 9).

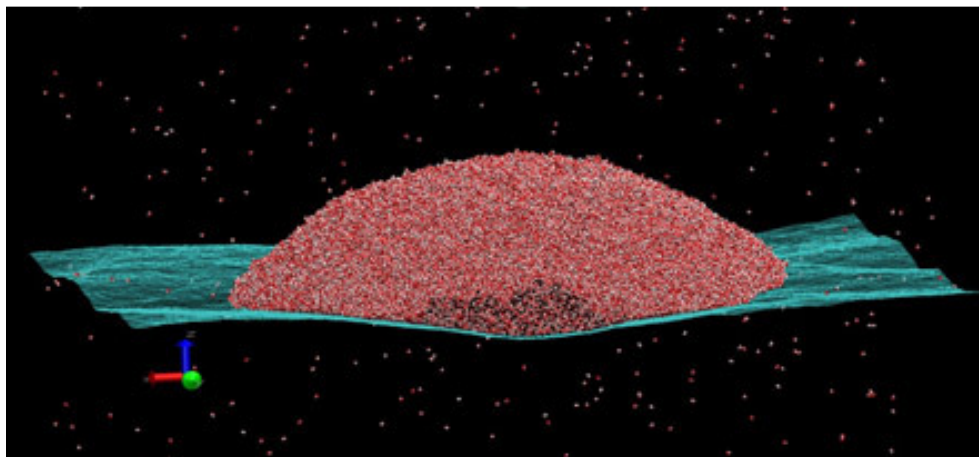


Figure 12: An evaporating nano-droplet on a black silica surface simulated with MD simulations. The total system consists of a million atoms. Simulations were carried out on a super computer cluster in NSF, using 256 CPUs.

In addition, a specific tool known as CHARMM (Chemistry at HARvard Macromolecular Mechanics: force fields for molecular dynamics simulation) will be included to model the nucleic acid hybridization phenomenon. The integrated simulation platform will allow us to model the process of the hybridization events taking place in our evaporation droplet detection system.

Aim 3: Verification of the device efficiency, repeatability, accuracy, and utility.

Task 3-1: Validation of the core technology using synthesized DNA and RNA molecules.

The team will design and order synthesized target DNAs and RNAs to evaluate the sensitivity and specificity of the technology using spiked samples. The molecular length will be between 20mers to 200mers to cover the DNA and RNA fragments for diagnosis. We will also use synthesized DNAs with 1, 2, 3, and 4 mismatched pair to evaluate the specificity and the effectiveness of algorithms to distinguish specific and non-specific binding. The device will be formed in a 2D array format, consisting of multiple probes for different target molecules as well as on-chip negative control. For each probe, there will be multiple repeats to assess the repeatability of the results and statistical variations.

Initially the synthesized DNA and RNA molecules will be spiked in diluted buffer so the sample can be directly dispensed to the device as droplets. After the process is well established and characterized, the same DNA and RNA molecules will be spiked in serum and urine samples and then use commercial nucleic acid extraction kits to recover the spiked DNAs and RNAs. Since the synthesized DNAs and RNAs cannot be

found in human serum or urine, one can perform the tests in conditions closer to the real samples while the results can be precisely quantified and evaluated.

Task 3-2: Validation of the technology by detecting bacteria DNAs.

The objective is to validate the device and methodology by detecting 16s rRNA gene sequencing for *chlamydia trachomatis* (CT, pathogen for sexual transmitted disease) identification. The result will be validated with separate technology platforms such as qPCR and perform systematic experiment to remove factors that could affect the accuracy and repeatability of the test.

Trichomonas vaginalis Donne (ATCC® 30001D™) will be used for the proposed work. Parasites will be grown in Trypticase-yeast extract-maltose (TYM) medium with 10% heat inactivated horse serum and incubated at 37°C. DNA will be extracted from specimens using the automated MagNA Pure LC Instrument (Roche Diagnostics). For DNA extraction with the MagNA Pure LC, the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics) will be used. Initially, sample suspensions will be transferred into Eppendorf cups containing glass beads (1,180 microns; Sigma, Deisenhofen, Germany) and vortex thoroughly. Then, 200 µL of the supernatant will be pipetted into the MagNA Pure LC sample cartridge. In the automated DNA isolation process, the samples are dissolved and simultaneously stabilized by incubation with buffer containing guanidinium thiocyanate and proteinase K. Total nucleic acids will be bound to the surface of glass magnetic particles, and unbound substances will be removed by several washing steps, and purified DNA will be eluted with a low-salt buffer. The genomic sequence of *Trichomonas vaginalis* has been analyzed and 10 probes (70 to 80 mers each) have been synthesized and anchored to the hydrophilic islands.

Risk Mitigation Plan:

The fabrication of black silica substrate and the nucleic acid enrichment by evaporating process have been accomplished and verified (Figure 2). Retention of target nucleic acids from total RNA or cDNA over 50% during the enrichment procedure needs to be confirmed using spiked, fluorescently labeled oligonucleotides with known initial concentration. If the target nucleic acids cannot be maintained during the evaporation process, the pH and ionic composition of the sample buffer will be modified to achieve better target nucleic acid retention.

If the simulation model does not accurately predict the detection performance, the macroscopic and microscopic components of the model will be decoupled and the team will utilize the experimental data as feedback to improve the platform. Furthermore, components of the simulation model will be examined and then substituted with alternative modules. Validation between the simulation and experimental data will facilitate the optimization of operation protocol and will determine the range and detection limit of the system platform.

Currently, the team has demonstrated detection sensitivity of the system at 10fM with a linear range of at least 3 orders of magnitude (figure 7). Further testing to ensure repeatability will be conducted. If the goal of <15% measurement variation is not reached, protocols to modify hybridization buffer, washing strength, and Q-dot labeling condition will be tested to minimize detection variation and to increase signal to noise (S/N) ratio. Specifically, hybridization conditions under various combinations of reagent parameters will be simulated and tested in order to achieve an optimized detection performance.

Aims	Milestones
Aim 1: Development of the technology platform for high sensitivity, accurate, and low cost detection of nucleic acid targets .	- Complete 3 design/fabrication/test iterations on hydrophilic micro-islands surrounded with hydrophobic black Si. The optimized devices will retain >50% target oligonucleotides with $\pm 15\%$ repeatability. - Enrich the oligonucleotide concentration from and detect fluorescently labeled nucleic acid molecules at $\geq 10\text{fM}$.
Aim 2: Development of physical models for rational design of the device and insights for the on-chip hybridization process.	- Build the simulation platform incorporating the Poisson-Boltzmann model and the effects of steric hindrance, Coulomb repelling force, and temperature. - Verify the model with experiment and obtain the parameters from experiment and/or molecular dynamic calculations - Build the modular dynamic simulation model for DNA/DNA and RNA/DNA hybridization under different conditions. - Combine the macroscopic and microscopic models into an integrated platform to analyze and predict the device behaviors.
Aim 3: Verification of the device efficiency, repeatability, accuracy, and utility	- Detecting and quantifying 16S rRNA Gene Sequencing for <i>chlamydia trachomatis</i> at 10fM concentration using device in Aim 1. - Validate simulation data with experimental results specifically within the window of 10fM to 1pM dynamic range (physiologically relevant range for many circulating bio-markers). - Characterize the device sensitivity and achieve $\pm 15\%$ repeatability and accuracy.

TIMELINES	Year 1				Year 2				Year 3			
Task 1-1	←			→								
Task 1-2		←			→							
Task 2-1			←		→							
Task 2-2				←				→				
Task 3-1				←					→			
Task 3-2						←						→