Abstract

Direct and indirect sensing of biological interactions using pH-sensitive silicon nanoscale field effect transistors

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Biosensors are playing an increasingly important role in gathering analytical data across various fields. From personalized monitoring, to food and water safety, to drug discovery, these tools are allowing human discoveries and quality of life to advance at an unprecedented pace. Nano-field-effect-transistors (nanoFET) are highly sensitive sensing elements with a multitude of benefits. They offer ease of integration with on-chip signal conditioning circuitry, and they are low power and low noise devices, which means they can be made portable. The measurement of macromolecular interactions is particularly useful in biosensing, however, the main drawback is that Debye screening in physiological buffers limits the applicability of FET sensors in real world macromolecular sensing applications. We overcome this challenge by translating the specific biorecognition event into a pH response, which, due to the small size of protons, is immune to screening. In this work, we use noise spectroscopy to drive the design of optimal nanoFET sensors, and demonstrate the application of this approach to measure meaningful macromolecular interactions.

Using low frequency noise measurements and modelling, we discuss that different applications have different sensor optimizations in terms of sensing dielectric and dimensions. For our application of pH sensing, we demonstrate that Al₂O₃ is the
preferred pH-sensing dielectric based on its higher signal to noise ratio, compared to SiO$_2$. We also briefly discuss scaling effects, and explain the rationale behind preferring larger devices when the trap density is equal.

We then use our nanoFETs in biologically-meaningful applications. Many enzyme-substrate interactions produce a pH change by altering the exposure of ionizable groups on the substrate. Using nanoFET sensors, these minute changes can be directly detected, even in the presence of buffering agents and physiological ionic strength. We were able to quantify substrate concentrations (urea) in its physiologically normal and elevated ranges (<200 µM to >20 mM) in phosphate buffered saline (PBS), and enzyme (penicillinase) activities as low as 0.02 units (~300 pM) in PBS and urine. Owing to the real time capability of nanoFET sensors, we were also able to accurately extract kinetic constants from enzyme-substrate interactions, as we demonstrated using acetylcholine and acetylcholinesterase. This platform allows for the direct detection of functional enzymes and metabolites in their native environments.

We also demonstrate an indirect application of this approach. Enzyme-linked immunosorbent assays (ELISAs) remain the gold standard in protein detection to date. However, due to the aforementioned benefits of electronic detection, it is advantageous to integrate nanoFET sensing for readout. This can be achieved by replacing the amplification enzyme in ELISA with a pH-changing enzyme, but the detection limit is ultimately limited due to linear amplification. DNA amplification is an exponential phenomenon that changes the solution pH as the reaction progresses, thus allowing for nanoFET readout. This not only can be applied to detect DNA concentration, but by replacing the enzyme in ELISA with a DNA strand then conducting amplification, we
can achieve ultrasensitive detection of proteins, surpassing ELISA results. We demonstrated this using the model system of IL-2 detection from serum, with detection limits on the order of 10 fM, through processes called immunoPCR and immunoLAMP. This method is universally applicable and can be expanded to include a wide variety of analytes.
Direct and indirect sensing of biological interactions using pH-sensitive silicon nanoscale field effect transistors

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To my parents
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“Piglet noticed that even though he had a very small heart, it could hold a rather large amount of gratitude” – Winnie the Pooh

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INTRODUCTION

The world of biosensing is ever-changing. From large diagnostic imaging machines such as magnetic resonance imaging, to small wearables such as fitness trackers, from in vitro platforms such as pregnancy sticks, to in vivo implantable devices, from electrical readouts such as impedance spectroscopy, to optical readouts such as fluorescence microscopy, biosensors have permeated various aspects of people’s lives to improve our understanding of biology, health, and quality of life. Biosensors are ubiquitous, but their developments are not without challenges. The sensor response often depends on the biochemical marker(s) of interest, yet the number of biophysical and biochemical signals worthy of measurement are vast and growing. An ideal sensor has good specificity, a good dynamic range encompassing the physiologically/biologically normal and abnormal (e.g. diseased) ranges, high sensitivity, excellent resolution and
integratability. Since these are important terms of sensor performance, a definition and explanation of each in the context of biosensing is provided in Box 1.

**Box 1**

**Specificity** – *the measure of the sensor’s ability to generate a positive response only to the target species*. In biomolecular sensing, specificity is often imparted by having a receptor that recognizes the target species and has low cross-reactivity to other molecules in the sample. A more specific receptor will often translate to a more specific biosensor.

**Dynamic range** – *the range of target analyte concentration over which there is a quantifiable variation in the sensor output*. The lower bound of the dynamic range is the low limit of detection (LOD), which depends on the signal to noise ratio (SNR) of the system. The upper bound of the dynamic range is often dependent on maximal receptor occupancy or instrumentation limit, and is often less of an obstacle in sensor design. Sample dilution can be an easy way to biochemically extend the upper limit of detection.

**Sensitivity** – *the amount of change in output signal per unit of change in input*. Sensitivity is not to be confused with the limit of detection, as it is purely a measurement of the effectiveness of signal transduction and does not take into account the noise level.

**Resolution** – *the smallest amount of change detectable*. Limit of detection is particularly important when measuring absolute biomarker concentration, but resolution is particularly important when measuring factors like pH, where the change away from a baseline is of concern. Resolution can be thought of as the limit of detection of change, and also depends on the SNR of the system.

**Integratability** – *the ability of a sensor to combine with upstream sample preparation modules, downstream signal processing modules, other sensing modalities or platforms, and itself in a multiplexed manner*. The ability to integrate the sensor with processing components allows for automation to reduce user error and miniaturization to enable portability. The ability to include other sensing modalities can offer a more accurate picture of the sample solution. The ability to multiplex can offer statistical meaningful measurements, and potentially multi-marker measurements.

The optimal sensor design and sensing modality ultimately depends on the intended application. Oftentimes, different sensors will offer different information (e.g.
mass, charge, size, reactivity, etc.). There is significant research effort in each sensor area that collectively builds the knowledgebase of biosensing.

Field effect transistor (FET)-based biosensors are low noise, low power, charge-sensitive devices that have been shown in a variety of biosensing applications. FETs are based on a purely electrical readout, hence their peripheral equipment can be miniaturized, making them more amenable to integration for portable applications. Since their performance is related to the charge density on the target molecule, they can sense extremely small molecules in solution, such as ions, that would be difficult to achieve with other sensing modalities. This is why over the years, pH measurements using ion-sensitive FETs have received overwhelming attention, and have gone on to commercialization. However, there are still several obstacles hindering the adoption of FETs for sensing larger molecules such as proteins.

In this thesis, I present some of the work done over the past several years, with focus on measuring macromolecular interactions such as protein recognition and enzyme-substrate reactions using pH-sensitive FETs as readout. My work builds on the vast resources discovered by both previous students in my lab, and also the sensing community. The work is done using silicon nanowire and nanoribbon FETs, herein referred to as nanoFETs, but is applicable to other pH-sensitive sensors. The thesis is structured as follows:

Section 2 discusses the working principles of the FET as a biosensor.

Section 3 introduces direct macromolecular detection and pH detection, and the motivation for using pH to sense macromolecular interactions.

Section 4 presents the fabrication flow and sensing setup.
Section 5 discusses signal to noise ratio. The theoretical and experimental considerations of signal generation by pH are presented, and noise analysis is shown. Various comparison parameters are calculated, which motivate certain design considerations.

Section 6 presents the use of pH to directly measure certain classes of enzyme-substrate interactions. We show examples of substrate concentration detection, enzyme activity detection, and kinetic constant quantification.

Section 7 presents the use of pH to indirectly measure protein and DNA concentrations, with main focus on protein measurement through immuno-polymerase chain reaction.

Section 8 presents an improvement to immuno-polymerase chain reaction with immuno-loop mediated isothermal amplification.

Section 9 summarizes the work presented in this thesis, and revisits the terms discussed in Box 1 in future outlook.
WORKING PRINCIPLE OF NANO FIELD EFFECT TRANSISTORS IN SENSING

A transistor is a three-terminal device where the resistance between two of the terminals is controlled by the voltage applied to a third terminal. Its name comes from the combination of the terms “transfer resistor”. The transistor was first patented by Lilienfeld in 1925, and the first working device was presented by Shockley, Brattain and Bardeen of Bell Labs in 1947. An important type of transistor is the metal-oxide-semiconductor field effect transistor (MOSFET), invented by Kahng and Atalla of Bell Labs in 1959 [1]. The MOSFET is a purely solid-state device, consisting of a semiconducting channel between the source and drain, and an insulating oxide between the channel and the metal (or polysilicon) gate. The semiconducting material is most commonly silicon, and the doping atoms are introduced in the structure to increase the number of charge carriers. Substituting silicon atoms in the lattice with acceptor atoms
such as boron introduces additional holes to make the material $p$-type, while substituting with donor atoms such as phosphorus introduces additional electrons, making the material $n$-type. Differently doped devices require different polarities of voltage control on the gate.

The sensing element used in the work presented in this thesis is a nanoFET, which is a class of ion-sensitive field effect transistors (ISFETs). ISFETs, originally introduced by Piet Bergveld in 1970 [2], are essentially MOSFETs without a top gate on the oxide. In the case of an ISFET, the gate dielectric is in direct contact with a test solution, and gating is achieved via a reference electrode inserted into solution (Figure 1). As the name implies, the conductance between the source and drain in the ISFET are influenced by the field effect resulting from ions from the test solution.

![Cross-sectional view of (a) a conventional MOSFET vs. (b) an ISFET.](image_url)

**Figure 1.** Cross-sectional view of (a) a conventional MOSFET vs. (b) an ISFET.
NanoFETs include nanowire, nanoribbon, and nanoplate field effect transistors. They share a common feature of nanoscale dimensions in the z-direction, hence their name. Unlike the traditional bulk MOSFETs, which rely on p-n junctions to isolate the devices from each other and bulk silicon, nanoFETs are typically isolated from each other by the removal of adjoining silicon, and isolated from the silicon handle by the buried oxide layer. This allows nanoFETs to have the same doping polarity throughout, and obviates the need for p-n junctions, which can significantly complicate the fabrication flow and contribute parasitic capacitances to the device. Figure 2 compares the cross-sectional view of a traditional bulk ISFET and a nanoFET. The detailed fabrication flow of nanoFETs will be presented in Section 4.1, but the main motivation in choosing nanoFETs as our sensing element comes from the ease of fabrication. The motivation for choosing either architecture due to sensing performance is not crystal clear, and excellent sensing responses have been achieved from both according to reports in literature [3, 4]. This is an important area of investigation into the future for this field, i.e. what device architecture, size, and materials yield the best performance sensor for a particular application?
In our work, we use nanoFET devices made from $p$-type silicon-on-insulator (SOI) wafers, which show conduction behavior when gate voltage ($V_{GS}$) is more negative than the threshold voltage ($V_T$). We will first introduce the theory of $p$-type MOSFETs, then describe how it is applied to ISFETs.

In MOSFETs, when the gate, dielectric, and semiconductor body are placed in contact, the difference in work functions between the metal and semiconductor, as well as oxide charges will both contribute to a band bending in silicon near the oxide-semiconductor interface. By applying a voltage to compensate for the built-in bias, one can negate the band bending effect and achieve flatband (Figure 3a). The required compensating voltage $V_{FB}$ is calculated by:

$$V_{FB} = \frac{\Phi_M}{q} - \frac{\Phi_S}{q} - \frac{\sigma_{ox}}{C_{ox}}$$

(1)

where $\Phi_M$ and $\Phi_S$ are the work functions of the metal (or polysilicon) gate and semiconductor, respectively, and $\sigma_{ox}$ and $C_{ox}$ are oxide charges and oxide capacitance.
per unit area, respectively. $\sigma_{ox}$ is a combination of fixed and mobile charges in the oxide. $q$ is elementary charge.

The different regimes of operation for a $p$-type metal-oxide-semiconductor structure relevant to nanoFET biosensor operation are described below. Note that since the channel and source drain regions are doped with the same polarity dopant, we do not typically use inversion as a mode of operation.

**Depletion:** In depletion, the transistor is turned off, and there is minimal conduction between source and drain. This occurs when a positive voltage is applied on the gate relative to the semiconductor, or a negative voltage smaller in magnitude than $V_T$ is applied, causing hole density near the surface to dip below bulk hole density, and downward band bending is observed (See Figure 3b).

**Accumulation:** In accumulation, the transistor is on, and the holes are drawn to form a conductive path between source and drain. This occurs when a negative voltage larger in magnitude than $V_T$ is applied on the gate relative to the semiconductor, causing hole density near the surface to increase, and an upward band bending is observed (see Figure 3c).

In the case of a nanoFET, the semiconductor is thin, typically comparable to the carrier screening length in the material; therefore, the band diagram for a nanoFET might look more like the figures in Figure 3 truncated before the bands flatten out to the right.
Figure 3. Band diagrams showing (a) flatband, (b) depletion, and (c) accumulation conditions of a metal-oxide-semiconductor structure through controlled gate voltage. \( E_c \), \( E_f \), and \( E_v \) are the conduction band energy, Fermi level, and valence band energy of the semiconductor, respectively, and \( E_{fm} \) is the Fermi level of the metal gate.

When the device is on at low drain voltages \( V_{DS} \), the drain current \( I_{DS} \) can be expressed as:

\[
I_{DS} = \mu C_{ox} \frac{W}{L} (V_{GS} - V_T) V_{DS}
\]  

(2)

where \( \mu \) is the carrier mobility, and \( W \) and \( L \) are the width and length of the channel. In accumulation, the threshold voltage \( V_T \) is equivalent to the flatband voltage \( V_{FB} \).
When the device is exposed to solution in an ISFET setup, an interface potential \( \psi_0 \) develops at the dielectric-solution interface, which is a function of surface, solution, and reference electrode characteristics. Thus, the \( V_T \) term in Equation 2 can be expressed as:

\[
V_T = \frac{\Phi_{El}}{q} - \psi_{El} - \psi_0 + \chi^{\text{mol}} - \frac{\Phi_{Si}}{q} \frac{\sigma_{ox}}{C_{ox}}
\]  

(3)

\( \Phi_{El} \) is the work function of the reference electrode, and \( \psi_{El} \) is the potential drop at the reference electrode-solution interface. For a true reference electrode (See Section 4.5.2), this potential drop is constant. \( \chi^{\text{mol}} \) is the surface dipole moment of the solution, which is also generally regarded to be constant. Therefore, in the case where gate and drain voltages are fixed, \( \psi_0 \) is the only variable term in Equation 3. \( \psi_0 \) is a function of chemical changes at the surface of the dielectric, and can change with pH, or binding of small molecules, proteins, nucleic acids, and cells. Thus, monitoring \( \psi_0 \) changes via the change in \( I_{DS} \) is an effective way of probing chemical changes at the dielectric-solution interface. Figure 4 presents a typical current vs. voltage relationship for a \( p \)-type nanoFET device. The device is depleted at voltages above \( V_T \). As the gate voltage becomes more negative, the device turns on as charges accumulate in the channel. Within the gating range, the leakage current \( I_{GS} \) remains in the tens of pA range, showing good electrical isolation between the channel and solution.
Figure 4. Current vs. voltage relationship for a typical nanoscale field effect transistor.

Since a change in $\psi_0$ is reflected as a change in $V_T$ of the device (Equation 3), we can see this in the $I_{DS}$ vs. $V_{GS}$ relationship as a shift in the curve (schematically represented in Figure 5a). If we set the operating point of this device at a specific gate voltage in accumulation, $I_{DS}$ can be monitored in real time (schematically represented in Figure 5b). The change in $I_{DS}$ can be converted back to the change in $\psi_0$ using the slope of the $I_{DS}$ vs. $V_{GS}$ relationship, called the transconductance. The transconductance ($g_m$) is defined as:

$$g_m = \frac{\partial I_{DS}}{\partial V_{GS}}$$

(4)

For $p$-type nanoFETs operated in accumulation, $g_m$ is a negative value.

Since $\Delta \psi_0$ is not a function of device dimensional characteristics, and is rather reflective of only the equivalent threshold shift caused by biochemical changes at the
dielectric surface, we found this to be an effective way to calibrate the devices so that comparisons between measurements on different devices can be made [4-6]. This yields:

$$\Delta \psi_0 = \frac{I_{DS} - I_{DS0}}{g_m}$$

(5)

Where $I_{DS0}$ is typically the baseline current before the biochemical change takes place, or current at a certain designated time $= 0$.

Figure 5. Schematic representation of (a) a shift in threshold voltage as a result of a change in surface potential (black is the original curve and red is the curve after change), and (b) real time change in drain current as a result of this event.

Charges at the dielectric-solution interface are completely screened by ions in the solution double layer and carriers in the silicon, thus the capacitance model is that of a charge-sharing one, with the solution capacitor in parallel with the dielectric-silicon capacitor stack, as shown in Figure 6a. $C_{Si}$, and $C_{dl}$ are the capacitances per unit area of the silicon channel and solution double layer respectively. An example of charge distribution involved in sensing is provided in Figure 6b. If the biochemical changes at the oxide-solution interface is negative in charge, positive ions will accumulate in solution, and holes will accumulate in the nanoFET channel, such that under equilibrium,
the total effect of the counter-charges completely screen out the change in charge at the oxide-solution interface. The induced counter-charges in the channel are sensed as drain current when $V_{ds}$ is applied.

![Diagram](image)

**Figure 6.** (a) Charge-sharing model of capacitances in the nanoFET sensor. (b) Example of charge distribution involved in sensing (not to scale).

This charge-sharing model in Figure 6 gives us the following relationship for charge density ($\sigma_0$) at the dielectric-solution interface:

$$\sigma_0 = C_{dl}\psi_{dl} + \frac{C_{ox}C_{Si}}{C_{ox} + C_{Si}}\psi_0$$

(6)

where $\psi_{dl} = \psi_0 - \psi_{bulk}$. Here, $\psi_{bulk}$ denotes the bulk potential of the solution, which is equal to the applied gate voltage minus potential drops at the gating electrode-solution interface. When the gating is applied via a reference electrode, the interface potential drop should be constant (see Section 4.5.2), thus yielding a constant $\psi_{bulk}$ for a particular applied gate voltage. Therefore, we do not include the double layer capacitance at the
gate-solution interface in our model in Figure 6a. Since most of our measurements are carried out in the strong accumulation regime, we simplify the above equation by assuming $C_{Si} \gg C_{ox}$, hence, the above equation reduces to $\sigma_0 = C_{dl} \psi_{dl} + C_{ox} \psi_0$. Since $C_{dl} \gg C_{ox}$ due to $\varepsilon_{dl} \gg \varepsilon_{ox}$ (relative permittivities of double layer and oxide), we can further simplify the surface charge relationship to just:

$$\sigma_0 = C_{dl} \psi_{dl}$$  \hspace{1cm} (7)$$

The above model allows us to assume that the change in charge at the dielectric-solution interface is mostly screened by the double layer in solution, although it is important to note that in reality, there will always be a small amount of charge that is being screened by the nanoFET channel. The magnitude of charge buildup in the nanoFET compared to in solution is determined by the ratio of $C_{ox}$ to $C_{dl}$, and any change in $\sigma_0$ at the interface redistributes charges in the nanoFET and solution according to this ratio. This forms the basis for nanoFET sensing in general.
3.1 Direct Charge Sensing

NanoFET-based sensing has been demonstrated thus far mostly in direct sensing platforms [6, 7]. This requires functionalizing the dielectric surface with a receptor that specifically recognizes the target analyte. For DNA, this receptor can be a complementary strand [8, 9], or a DNA-binding protein [4]. For an antigen, this receptor can be an antibody [10, 11]. When the analyte binds to the localized receptors at the dielectric-solution interface, the change in the charge state at the interface induces a change in the conductance in the underlying nanoFET device. This sensing modality can achieve real-time, label-free, and sensitive detection of highly charged molecules in solution.
Direct sensing relies on the target molecules being charged in solution, which is often not an issue, as majority of biomolecules have ionizable groups on their surface that can protonate or deprotonate depending on the pH of the solution. Nevertheless, the larger the difference between the isoelectric point of the molecule and the pH of the solution, the more strongly charged the molecule is, and the easier it is to detect.

Despite the significant promise demonstrated using direct detection, there are several issues hindering its widespread adoption:

3.1.1 Debye Screening

In any ionic solution, the introduction of a charged surface will cause nearby ions to redistribute, thus screening the charges. Opposite charges will be attracted close to the surface, and like charges repelled. This rearrangement of ions leads to an exponentially decaying potential from the surface into the neutral bulk of the solution. This ionic layer at the surface is termed the double layer, which consists of an inner immobile adsorbed layer called the Stern layer and an outer loosely adsorbed Diffuse layer. The effectiveness of surface charge screening is described by a characteristic length called Debye length ($\lambda_D$), which decreases with ionic concentration. Debye length is the length scale over which the potential decreases by 1/e, and is calculated by:

$$\lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r kT}{2 N_A q^2 I_c}}$$  \hspace{1cm} (8)
\( \varepsilon_0 \) is the vacuum permittivity, \( k \) is the Boltzmann constant, \( T \) is the temperature, \( N_A \) is Avogadro’s number, and \( I_c \) is the ionic concentration.

The rate at which the potential decays away from the charged surface is illustrated in Figure 7. The higher the ionic strength of the sample, the quicker the potential decays. \( \lambda_D \) values for several ionic concentrations of a monovalent electrolyte solution are collected in Table 1.

**Table 1. Debye lengths for different ionic strength solutions.**

<table>
<thead>
<tr>
<th>Ionic strength (mM)</th>
<th>( \lambda_D ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>0.79</td>
</tr>
<tr>
<td>15</td>
<td>2.51</td>
</tr>
<tr>
<td>1.5</td>
<td>7.93</td>
</tr>
<tr>
<td>0.15</td>
<td>25.07</td>
</tr>
</tbody>
</table>

Physiological fluids such as blood have ionic strengths around 150 mM NaCl, which has a very short Debye length (0.79 nm, see Table 1). The dashed magenta line crosses each potential decay curve at the Debye length (Figure 7). For detection of molecules that are highly charged and have small receptors (e.g. nucleic acids), direct detection in high ionic strength buffers is possible [12]. However, for protein antigen detection, full size antibodies are typically on the order of 10 nm (as illustrated in Figure 7), therefore, unless the sample is sufficiently dilute in ions, the binding of the antigen at that distance from the nanoFET surface is virtually undetectable [13].
Figure 7. Surface potential decay as a function of distance from charged surface at different ionic strengths. The size of a typical antibody molecule is drawn for reference.

As a result, majority of the work in this area has been proof of concept studies using protein standards spiked into diluted buffers [4, 14], or physiological samples desalted using complex procedures [10, 15]. This is not widely applicable as protein stability and protein interactions can be disrupted under non-optimal salt concentrations [16, 17]. Other methods such as engineering smaller receptors (e.g. antibody fragments or aptamers) [11, 18, 19], or effectively extending the Debye length at the device surface by functionalization of polyelectrolytes [20] have been presented, which shows a promising direction for future research. However, these solutions have only been demonstrated in a few reports and have not been widely adopted to solve this issue of Debye screening.

3.1.2 Device Functionalization

Due to the limitations caused by Debye screening, successful measurement requires analyte binding as close to sensor surface as possible, which puts stringent requirements on surface functionalization. Ideally, the surface would be covered by a
medium density of receptor molecules attached to the surface via a monolayer linker. If the surface coverage is too low, too few target molecules will bind, decreasing the potential signal. If the surface coverage is too high, the receptor-analyte pair does not have enough angular freedom to move close to the surface, thereby also decreasing the potential signal [11]. If the functionalization quality is poor and the reactants crosslink in an undesired manner, the functionalization may not form a monolayer, therefore the analyte may be forced to bind farther away from the surface, further decreasing the potential signal. The difficulty in achieving repeatable functionalization between different research groups also makes it difficult to compare data across publications.

3.1.3 Competing Signals

The biochemical and electrical changes occurring at the surface during binding in solution are complex and difficult to verify. Since functionalization of a dielectric with large receptors can never fully deplete the hydroxyl groups on its surface, there are at least two types of species whose interaction with the surface will influence $\psi_0$ – the analyte, and protons. During the binding of the target molecule to the surface, the charges on the ionizable groups at the dielectric-solution interface can induce a potential shift. However, this localization of high concentrations of ionizable groups can also cause a change in the surface solution pH. This change in the local pH can be buffered by the dielectric surface, causing a shift in the protonation equilibrium at the surface, which again tends to change $\psi_0$. These two forces on $\psi_0$ oppose each other, and can cancel out for surfaces with near-Nernstian pH behavior [21]. This is part of the reason why despite the great success of pH sensing on high-k dielectrics like $\text{Al}_2\text{O}_3$ and $\text{Ta}_2\text{O}_5$, majority of
direct charge sensing measurements have been performed on low pH-sensitivity surfaces like SiO$_2$. Even with low pH-sensitivity surfaces, the signal is subdued as a result of competing reactions, thus hindering the sensitivity, limit of detection, and the resolution of the sensor.

In addition to competing surface reactions, competing signals could also cause difficulty in interpreting measurement results. Different macromolecule concentrations spiked into diluted buffers often cause a slight change in pH that could be misinterpreted as a binding signal. When the ionic strength is high, this pH change occurs almost instantaneously upon solution exchange, therefore can be decoupled from slower binding events. However, since direct sensing often requires low salt solutions due to Debye screening concerns, this juxtaposition of signals becomes more difficult to decouple.

Given the exceeding number of confounding factors in direct analyte sensing, to move forward in this direction, we must emphasize the use of a differential setup in order to ensure that observed signals are due to real binding events and not pH changes, nonspecific adsorption, or just drift. In order to specifically detect the molecule of interest, a number of devices on the same chip must be functionalized with a similar, but non-cross-reactive receptor. To enable differential functionalization, we have designed a removable microfluidic channel to isolate different groups of devices, as shown in Figure 8.
Figure 8. (a) Mask design showing overlay of chip containing devices and microfluidic channel for functionalization. (b) Packaged chip showing microfluidic channel over wirebonded devices. (c) Fluorescent image of different dyes (green: fluorescein isothiocyanate (FITC), yellow: tetramethylrhodamine (TAMRA) flown into alternating channels, showing isolation of solutions between channels.

Different solutions can be introduced to different channels containing different nanoFET devices to achieve differential functionalization. After functionalization, the microfluidic channel is removed by soaking the entire chip in phosphate buffered saline (PBS) to prevent cross contamination. The chip is then quickly dipped in deionized (DI) water and dried in a stream of nitrogen. We then place a single channel microfluidic chip over all the devices and introduce our experimental solutions. We have also tried multiple variations of this procedure, e.g. keeping the captured antibodies wet throughout the process and using a large microchamber to introduce turbulent flow.

Nevertheless, as a result of the aforementioned factors, despite successful fluorescence attempts that show functionalization of our SiO$_2$ nanoribbon devices, we have had little success in being able to electronically sense direct, specific protein binding. An example is shown below, where four devices are functionalized with non-fluorescent neuropeptide Y (NPY) antibodies, and three devices are functionalized with a nonspecific
fluorescent antibody (FITC-anti-troponin) on the same chip. The success of the functionalization is determined by fluorescence, which should be stronger for the anti-troponin-functionalized devices compared to the anti-NPY-functionalized devices. Despite the high background autofluorescence from the SU-8 passivation layer on the chip’s surface, it is clear that the anti-troponin functionalized devices are indeed brighter than the anti-NPY functionalized devices, as shown in Figure 9. Since anti-NPY and anti-troponin are functionalized the same way, one can reasonably deduce that we have successful anti-NPY functionalization as well.

![Figure 9. comparison between nonfluorescent anti-NPY-functionalized devices (left) and FITC-anti-troponin-functionalized devices (right).](image)

However, the success in probe functionalization did not seem to translate into success in detection, as both the specific and control devices showed the same response to NPY solutions flown over the entire chip (Figure 10). This particular chip was measured on an eight-channel setup using seven viable devices (see Section 4.4.2 for details).
Figure 10. Sensing result of differentially functionalized chip (anti-NPY as specific antibody and anti-troponin as control antibody), with different concentrations of NPY antigen dissolved in 0.1x artificial sweat (AS) flown over the entire chip.

3.2 pH-based Sensing

In contrast to direct charge sensing, pH sensing has several advantages. pH changes are based on activity changes of protons, which are small in size, and therefore immune to the effects of Debye screening. In fact, pH changes can be measured under physiological ionic strengths, where the high salt concentration helps to stabilize the signal and reduces noise coupling from the environment. pH changes require no functionalization on the sensor to detect, and instead relies on natural dangling bonds at the dielectric surface, thereby obviating these challenges associated with functionalization. In addition, nonspecific adsorption can be more effectively blocked out for pH sensing due to the dramatic size differences between protons and adsorbing agents.
(typically macromolecules), thereby decreasing competing reactions, as well as the biological noise of the measurement.

Due to these benefits, pH-based detection is much more robust compared to direct macromolecular detection. Some interesting biological phenomena directly produce pH changes as a response. For example, T-cell activation can lead to a change in the rate of extracellular acidification, which can be used to measure antigen-specificity in T-cell populations [22]. Also, nucleic acid synthesis has been shown to acidify low-buffering media, which has been used to realize sequencing [3] and DNA amplification [23] measurements. Moreover, some biomolecular interactions, such as certain classes of enzyme-substrate interactions (described in Section 6), produce a pH change during alteration of the substrate molecule [5]. Therefore, measurement of pH changes in the presence of these events can be a simple yet reliable way to quantify them. However, not all interesting biological interactions directly produce pH changes. In those cases, an indirect mechanism can be employed to convert the biorecognition event into a pH response. For example, antibody-antigen binding can be converted to pH via an electronic enzyme linked immunosorbent assay (ELISA) [24-26], immuno-polymerase chain reaction (immune-PCR) (described in Section 7), or immuno-loop mediated isothermal amplification (immuno-LAMP) (described in Section 8).
Since the introduction of nanowire biosensors by the Lieber group [27], many different techniques for their fabrication have been proposed. These techniques can be mainly grouped into two categories: bottom-up and top-down. Most of the earlier works in the field relied on bottom-up methods to chemically grow nanowires, followed by their harvest and reassembly on a working substrate [28-31]. These nanosensors showed extraordinary device performance owing to their high crystallinity and smooth surfaces. However, they suffered from difficulty in integration and commercial scale production. Therefore, researchers have devoted significant attention into top-down fabricated nanosensors to create devices with well-defined dimensions and uniform electrical
characteristics [32, 33]. Compatibility with CMOS technology allows these sensors to be potentially designed into integrated circuits, along with addressing elements, low-noise amplifiers, and analog-to-digital converters. Having uniform device characteristics is especially useful for simultaneous measurements over multiple devices, where devices with similar threshold voltages allow the use of a global gating scheme [33]. Both nanoribbon and nanowire sensors have been defined using either optical or electron-beam lithography on ultra-thin silicon-on-insulator wafers, with nanoribbons being much cheaper and simpler to fabricate.

4.1 Nanoribbon Device Fabrication

For the nanosensor devices fabricated in-house, we opted for ribbon or even plate like devices with large dimensions and relaxed fabrication requirements. Since pH measurements yield a signal that scales with device area, the larger dimensions are actually preferable for our applications [34, 35] (See Section 5.4 for more details). A set of 8 optical lithography masks is used to precisely define the sensor locations and dimensions. The wafer designs are based on devices previously fabricated by our lab [36-38], but with some alterations to improve the uniformity and yield across the wafer. The fabrication process is as follows, also shown in Figure 11. More information about the features on each mask layer can be found in Appendix A. All lithography steps are carried out using an EVG 620 aligner for exposure.

1. **SOI.** The silicon-on-insulator (SOI) wafer we use for fabrication (SOITEC) starts off with a 70 nm top active silicon layer on a 145 nm buried oxide layer. The buried oxide layer provides electrical isolation from the silicon handle layer. The active silicon has a
resistivity of 8-12 Ωcm, and the silicon handle layer has a resistivity of 8-22 Ωcm. The starting wafer is shown in Figure 11 as step 1.

2. **Cleaning and active layer thinning.** The fabrication process begins with cleaning and thinning of the active silicon layer. The wafer is first RCA cleaned in a three step process \[39\]. Then, the wafer is dry oxidized in a furnace at 1100 °C to thin the active layer, and the grown oxide is etched away with a 10:1 buffered oxide etch (BOE). The oxidation times are designed with the preferred dielectric in mind, such that the final silicon channel height is approximately 40 nm, the Debye length in silicon with a carrier concentration of \(10^{16}/\text{cm}^3\). This is shown in Figure 11 as step 2.

3. **Alignment marks etching.** Mask #1 is used to define alignment marks on the wafer using Shipley S1818 photoresist, and reactive ion etching (RIE) based on \(\text{SF}_6/\text{C}_4\text{F}_8\) chemistry (Oxford 100) is used to etch alignment marks 2 μm deep into the wafer. The \(\text{SF}_6/\text{C}_4\text{F}_8\) chemistry etches silicon at a rate of \(~\)120 nm/min, and \(\text{SiO}_2\) at a rate of \(~\)600 nm/min.

4. **Backgate etching.** Backgate access is opened from the top to enable facile dry characterization of devices. Mask #2 is used to define vias to the Si substrate using Shipley S1818 photoresist, and the \(\text{SF}_6/\text{C}_4\text{F}_8\) chemistry is used to etch through the active silicon and the buried oxide. 10:1 BOE is used to ensure that the Si substrate is exposed.

5. **Implantation.** Implantation is required to ensure ohmic contacts between the Si and the metal electrode, so that device operation is not dominated by contact resistance. Mask #3 is used to protect the entire wafer with Shipley S1818 photoresist, except for backgate and source/drain areas for the nanoribbons. The wafer is sent out of house (either to Cornell NanoScale Science and Technology Facility (CNF) or Leonard Kroko for BF$_2^+$-
implantation at 10keV and a dose of $2 \times 10^{15}$/cm$^2$ with a 7° tilt. The off-axis implantation direction causes scattering of ions with the silicon lattice to prevent channeling of dopant ions inside the silicon. This is shown in Figure 11 as step 3.

6. **Nanoribbon definition.** After implantation, Mask #4 is used to define nanoribbons in the active silicon. Since this mask has a minimum nanoribbon widths of 1 µm, hexamethyldisilazane (HMDS) is spun on the wafer as an adhesion layer prior to Shipley S1805 photoresist. The ribbon definition is completed using RIE based on Cl$_2$ chemistry. This is shown in Figure 11 as step 4.

7. **Dielectric growth/deposition (and dopant drive-in).** After nanoribbons are etched, the entire wafer is RCA cleaned again, and either sent to CNF for atomic layer deposition (ALD) of Al$_2$O$_3$, or loaded into our cleanroom furnace for dry oxidation of SiO$_2$ (at 1100 °C) to form the 20 nm thick sensing dielectric. This is shown in Figure 11 as step 5. Al$_2$O$_3$ is a conformal layer covering the wafer whereas SiO$_2$ is only grown on the devices. This is the most important step in the fabrication process, as a good quality dielectric and interface will ensure low device noise, low hysteresis, high surface group density, high stability, and low current leakage when operated in solution. In the case of Al$_2$O$_3$, thermal annealing (900 °C, 10 minutes) is carried out afterwards to improve the dielectric-semiconductor interface. The thermal treatment from either annealing or oxidation also serves as a drive-in process to distribute the dopant ions and incorporate them into the lattice, so that they can ionize and contribute to conduction. The high temperature volatilizes the fluorine, and results in a boron doping concentration of $\sim 10^{19}$/cm$^3$ in the exposed regions to ensure ohmic contacts to the metal.
8. **Contact clearing.** Mask #5 is used to expose the source/drain and backgate contacts in Shipley S1805 photoresist, and 10:1 BOE is applied to clear out the dielectric in those regions to prepare for metal deposition. This is shown in Figure 11 as step 6.

9. **Metallization.** Two metal layers are subsequently deposited by lift-off using lift-off resist LOR 5A and Shipley S1805 resist for patterning. The first layer is 200 nm thick aluminum, defined by Mask #6, and includes all the contacts to devices and to backgate. After aluminum deposition, annealing was carried out at 460 °C for 20 minutes to allow aluminum to diffuse into the doped silicon and form an ohmic contact. This is shown in Figure 11 as step 7. Contact resistance is checked by measuring between two backgate contacts on the wafer. The second layer is 100 nm thick platinum, defined by Mask #7, which forms the on-chip solution gate electrodes. This layer is not included on all wafers as most of the intended applications are in pH-sensing, and platinum is a poor reference electrode for pH. A few attempts were made to fabricate on-chip Ag/AgCl electrodes using this mask, however, we found that the formed AgCl often flaked off from the electrodes to interfere with sensing.

10. **Passivation.** To prevent leakage current from solution into the device, a 2 μm thick SU-8 layer (SU-8 2002) is used to cover all exposed metal using Mask #8, opening only a small via over the nanoribbon device to be exposed to solution. This is shown in Figure 11 as step 8.
Figure 11. Fabrication process flow for silicon nanoribbon biosensors

Figure 12 shows a side by side presentation of the mask design for a 20 µm long by 3 µm wide nanoribbon device (Figure 12a), as well as an optical microscope image of the fabricated device itself (Figure 12b). The mask design for a single 6.6 mm by 6.6 mm chip (Figure 12c), as well as for the entire 4” wafer (Figure 12d), is also shown.
4.2 Challenges in Nanoribbon Fabrication

During the fabrication process, there were several challenges encountered:

4.2.1 Dielectric Window Opening

The removal of dielectric completely from the source drain regions and the backgate contacts is vital to ensuring an ohmic contact between the metal and the silicon.
We use the Nanospec Reflectometer to measure the oxide thickness in the backgate regions to track how much oxide we have removed by BOE etching. Unfortunately, since the source and drain contacts are much smaller, we cannot directly measure oxide thickness in those regions. However, the removal of oxide is much slower in the smaller regions. Therefore, it is important that the etch time be significantly prolonged even after the backgate contacts have been cleared to ensure that the source and drain regions are also cleared. We found that extending the etch time by approximately 1 min after the backgate contacts are cleared will also ensure that the source and drain regions are cleared. It is also critical that the alignment between the etch windows and the underlying silicon has sufficient tolerance that overetching does not result in punch-through of the buried oxide and shorting of the device.

4.2.2 SU-8 Passivation

Due to the fact that SU-8 is a negative photoresist, it is very difficult to remove once it has been exposed and crosslinked. Therefore, there is only one chance to successfully define opening vias in the passivation layer. If the SU-8 layer is overexposed, the opening window may not clear completely, leaving SU-8 residue over the nanoribbons, as shown in Figure 13. However, it is not easy to correctly estimate the exposure time needed during lithography, as the underlying structures and materials will largely influence the time needed. Therefore, we recommend using dummy wafers to dose test this lithography step before performing it on the wafer of interest. Our dummy wafers are made by depositing a 40-60 nm thick layer of polysilicon on a wafer with 145 nm of SiO₂ using plasma-enhanced chemical vapor deposition (PECVD). These dummy
wafers are used to test out every processing step along the way such that by the
passivation step, the dummy wafers have the same structures and similar materials as the
wafer of interest. Having appropriate dummy wafers for the passivation step significantly
improves the chances of successful lithography.

Figure 13. SU-8 residue in nanoribbon passivation opening

4.3 Nanowires

During the recent two years, we have had the opportunity to collaborate with a
molecular diagnostics company, QuantuMDx, who provided us with Al₂O₃-coated
nanowire FET sensors, so we did not have to continue our own fabrication runs. Each of
QuantuMDx’s nanoFET devices contains five nanowires in parallel to improve device
stability and uniformity, and each chip contains 64 devices in an arrayed format. They
were fabricated from 8” silicon-on-insulator wafers using a process similar to those
reported in literature [10, 40]. The devices are also p-type with buried oxide thickness
around 150 nm and active silicon layer thickness around 140 nm. Al₂O₃ was also
deposited as the sensing dielectric using ALD with a typical thickness below 15 nm. The
device source and drain are metallized with aluminum, and leakage current into solution
is suppressed with a passivating layer of SiO₂, with the Al₂O₃ over the sensors exposed.
An optical image of QuantuMDx nanowire sensors is shown in Figure 14a, with a single
device highlighted in a red box. The wires are too small to be seen in the image, but the
metallization to the five nanowires are clearly visible. A scanning electron microscope (SEM) image of a single nanowire is presented in Figure 14b.

![Figure 14. (a) Optical microscope images of QuantuMDx nanoFET sensor. The beige areas in the image are the source and drain metallization in aluminum, the blue areas are $\text{Al}_2\text{O}_3$, and the ochre areas are passivating $\text{SiO}_2$. (b) SEM image of a single nanowire.](image)

Although for our applications, larger area devices (i.e. nanoribbons) give a better signal to noise ratio compared to smaller devices (i.e. nanowires) with the same trap density (see Section 5.4 for more details), different fabrication processes can lead to different trap densities. The devices supplied by QuantuMDx were fabricated using state-of-the-art cleanroom facilities, and therefore showed excellent performance and uniformity across the chip. In addition, their devices are fabricated with an interfacial $\text{SiO}_2$ layer sandwiched between the $\text{Al}_2\text{O}_3$ and the Si channel, allowing them to take advantage of the near-Nernstian sensitivity of the $\text{Al}_2\text{O}_3$ as well as the low noise Si-$\text{SiO}_2$ interface [41].
4.4 Electrical Measurement Setup

All nanoFET data presented in this work has been taken using one of three setups, which evolved over the years: single-channel measurement setup [5, 42], eight-channel measurement setup [38], and 32-channel measurement setup [43]. The details of each setup are described below.

4.4.1 Single-channel Measurement Setup

In this setup, a dual-channel Keithley 2636 source meter is used to source gate and drain voltages to set the operating point of the device. Devices are wirebonded into 28 pin dual in-line packages (DIPs) (see Figure 15 for a packaged nanoribbon chip) to interface with the measurement setup. The drain current from the device is fed into a low noise current pre-amplifier (Stanford Research Systems 570), low-pass filtered with a cut-off frequency of 10 Hz (12 dB roll-off), amplified and converted into a voltage (typically 100 nA/V) to be measured by the data acquisition (DAQ) card (NI PCI-6251). A Labview program designed in-house monitors and records the signal in real time. Noise measurements were also taken using the single-channel setup, with the amplifier set to bandpass filter (0.03 Hz to 1 kHz, 12 dB roll-off).
4.4.2 Eight-channel Measurement Setup

An early iteration of a multi-device measurement setup allows up to eight devices to be measured simultaneously on a printed circuit board (PCB) [38], shown in Figure 16. Devices are wirebonded into 28 pin DIP packages to interface with the measurement setup (same as in Figure 15). Drain and gate voltages are supplied by the DAQ card (NI PCI-6251). The drain currents from eight devices are simultaneously fed into the inverting terminal of eight corresponding low noise op-amps (LT1012) configured in a low-pass circuit with a cutoff frequency of 1.6 Hz (R = 1 MΩ, C = 0.1 µF). The voltage at the readout is fed into eight channels of the DAQ card, which takes multiplexed readings from all eight devices. Board control and data recording are achieved via Labview programs. The circuit schematic for the eight-channel setup is shown in Figure 19a.
Figure 16. Photograph of eight-channel sensing setup.

4.4.3 32-channel Measurement Setup

The multi-channel setup was expanded to allow for the measurement of up to 32 devices on a PCB [43]. Devices are wirebonded into 68 pin leadless chip carriers (LCC) to interface with the measurement setup (see Figure 17 for a packaged chip). The LCC is inserted into the board, shown in Figure 18. Drain and gate voltages are supplied by the DAQ card (NI USB-6361). The drain current from the 32 device are simultaneously fed into the inverting terminal of eight quad low noise op-amps (LT1125). The op-amp circuit is designed to be a current-to-voltage converter with a gain of $10^6$ (1 V/µA) and a low pass filter with a cutoff frequency of 1.6 Hz ($R = 1$ MΩ, $C = 0.1$ µF). The resulting voltages are fed into a 32 channel multiplexer (ADG732BSUZ). The multiplexer
sequentially passes through the voltage output of each channel into the DAQ, and the
signal of each channel is individually monitored and recorded. Board control and data
recording are achieved via Labview programs. The circuit schematic for the 32-channel
setup is shown in Figure 19b.

Figure 17. Photograph of packaged nanoribbon chip in 68 pin LCC.

Figure 18. Photograph of 32-channel sensing setup.
Figure 19. Schematic of (a) eight-channel and (b) 32-channel measurement setups.

4.5 Liquid Measurement Setup

4.5.1 Fluidic Integration

Sample scarcity is often an issue for biological samples – in particular physiological samples. Therefore, being able to limit the required sample amount for analysis is advantageous. NanoFETs can be made into planar, high-density arrays, which are conducive to microfluidic integration. Two types of microfluidic setups are used in this work for sample delivery: reservoirs and microchannels. Reservoirs over devices are typically designed to hold a few to hundreds of microliters of sample, and sample exchange is done manually through pipetting. Reservoirs are typically made from polypropylene tubing and glued over the chip surface (Figure 20a). Drawbacks of the microwell method include evaporation of sample, which precludes long term measurements from being conducted on small samples, and potentially higher limits of detection due to the limited supply of analytes. However, open reservoirs allow for easier addition and mixing of reagents.
Microchannels are typically made via soft lithography using polydimethylsiloxane (PDMS). The dimensions of the channel are fully tunable based on the design of the mold, which is typically cleanroom fabricated using a negative resist like SU-8. The microchannel can be reversibly or irreversibly bonded to the chip surface using van der Waal’s forces or oxygen plasma activation, respectively. Photographs of packaged chips with microfluidic channels on them are shown in Figure 20b&c. Microchannels have micrometer dimensions, but may require more reagent overall due to the need for continuous flow. Flow-based delivery using a pump allows greater potential for system automation, and allows for more control in the fluidic delivery [44]. However, flow in microfluidic channels can be laminar, which can restrict mixing between fluids. Therefore, we chose to use reservoirs for solution delivery when looking at reactions that require mixing of solutions, and microchannels for solution delivery for measurement of solution pH sequentially.

Figure 20. Photographs of packaged nanoFET chips with fluidic integration. (a) Nanoribbon chip with reservoir. (b) Nanowire chip with microchannel. (c) Nanoribbon chip with microchannel.
4.5.2 Reference Electrode

Regardless of the measurement setup used, a reference electrode is required to set the solution potential at the desired operating point. An ideal reference electrode will have a negligible change in its surface potential in different solution states (in this case, under different solution pH) compared to the surface potential change at the nanosensor surface, and good stability over the measurement time. True reference electrodes are often bulky and difficult to integrate into the sensing setup, but if the sensing solution is known to have a constant chloride concentration, a Ag/AgCl wire can be used as a pseudo reference electrode. In open reservoir sensing, the solution gate electrode can be inserted directly into the open reservoir. However, in flow experiments, the reference electrode is typically integrated into the inlet/outlet tubing.

The Ag/AgCl wire is made by first soaking the Ag wire in nitric acid for 5 min to remove oxidized Ag on the surface, then rinsing in deionized water. The wire is then immersed in a solution of 100 mM KCl along with a platinum wire, and 2V is applied on the Ag in reference to the Pt. The current is monitored and the reaction is terminated when the current drops significantly from its initial value [45] (typically within 30 min for a Ag/AgCl wire electrode).
5.1 Signal: Mechanism of pH Sensing

The dependence of $\psi_0$ on pH is typically derived using the combined Gouy-Chapman Stern and site-binding models [46-50]. We solve this similarly to work presented in [51]. The dielectric surface participates in an acid-base reaction with the solution and changes $\psi_0$ depending on the pH. In the case of an oxide dielectric, the relevant reaction equations, in dissociation form, are:

$$
\begin{align*}
A - OH_2^+ & \rightleftharpoons K_a A - OH + H_3^+ \\
A - OH & \rightleftharpoons K_d A - O^- + H_3^+
\end{align*}
$$

This equilibrium is dictated by the concentration of protons near the surface ($H_3^+$), which is related to the bulk proton concentration ($H_b^+$) via the Boltzmann distribution:
\[ H_{s}^{+} = H_{b}^{+} \exp \left( -\frac{q \psi_{dl}}{kT} \right) \]  \hspace{1cm} (10)

with \( pH = -\log[H_{b}^{+}] \).

From Equation 9, we obtain the equilibrium constants for the two dissociation reactions:

\[
K_{a} = \frac{[A - OH][H_{s}^{+}]}{[A - OH_{2}^{+}]} \\
K_{b} = \frac{[A - O][H_{s}^{+}]}{[A - OH]} \]  \hspace{1cm} (11)

Since all surface sites are either protonated, neutral, or deprotonated, the total binding site density \( N_{s} \) is given by

\[
N_{s} = \left[ [A - OH_{2}^{+}] + [A - OH] + [A - O^-] \right] \]  \hspace{1cm} (12)

The difference between the protonated and deprotonated groups then gives rise to the surface charge density \( \sigma_{0} \). Therefore, this yields the relation:

\[
\sigma_{0} = q\left( [A - OH_{2}^{+}] - [A - O^-] \right) \]  \hspace{1cm} (13)

Combining Equations 11-13, we obtain the following relationship for surface charge density:

\[
\sigma_{0} = qN_{s}\left( \frac{H_{s}^{+} - K_{a}K_{b}}{H_{s}^{+} + K_{a}H_{s}^{+} + K_{a}K_{b}} \right) \]  \hspace{1cm} (14)

Solving for \( [H_{s}^{+}] \) in Equation 14 using the quadratic formula yields:
\[
\left[ H_s^+ \right] = \frac{\sqrt{K_a K_b} \left( \frac{\sigma_0}{2qN_s} \sqrt{\frac{K_a}{K_b}} - \sqrt{1 + \left( \frac{\sigma_0}{2qN_s} \sqrt{\frac{K_a}{K_b}} \right)^2 - \left( \frac{\sigma_0}{qN_s} \right)^2} \right)}{1 - \frac{\sigma_0}{qN_s}}
\] (15)

Using Equation 10, we can obtain a relationship between bulk proton concentration and surface charge density:

\[
\left[ H_b^+ \right] = \frac{\sqrt{K_a K_b} \exp\left( \frac{q\psi_{dl}}{kT} \right) \left( \frac{\sigma_0}{2qN_s} \sqrt{\frac{K_a}{K_b}} + \sqrt{1 + \left( \frac{\sigma_0}{2qN_s} \sqrt{\frac{K_a}{K_b}} \right)^2 - \left( \frac{\sigma_0}{qN_s} \right)^2} \right)}{1 - \frac{\sigma_0}{qN_s}}
\] (16)

Since we already have an expression for \( \sigma_0 \) in terms of potential across the double layer, \( \psi_{dl} \), from Equation 7, we can substitute that into Equation 16 to yield an explicit relationship between solution proton concentration and \( \psi_{dl} \).

\[
\left[ H_b^+ \right] = \frac{\sqrt{K_a K_b} \exp\left( \frac{q\psi_{dl}}{kT} \right) \left( \frac{C_{dl} \psi_{dl}}{2qN_s} \sqrt{\frac{K_a}{K_b}} + \sqrt{1 + \left( \frac{C_{dl} \psi_{dl}}{2qN_s} \sqrt{\frac{K_a}{K_b}} \right)^2 - \left( \frac{C_{dl} \psi_{dl}}{qN_s} \right)^2} \right)}{1 - \frac{C_{dl} \psi_{dl}}{qN_s}}
\] (17)

The solution pH is then just \(-\log[H_b^+]\).

Since \( \psi_{dl} = \psi_0 - \psi_{\text{bulk}} \), and \( \psi_{\text{bulk}} \) is fixed by the applied solution gate voltage, changes in \( \psi_{dl} \) is equal to changes in surface potential \( \psi_0 \). Using MATLAB, we can plot the derivative of the pH-surface potential relationship \( \frac{d\psi_0}{dpH} \) to visualize the sensitivity of different dielectric materials. The parameters that vary for different dielectrics include...
$K_a, K_b$, and $N_s$. The effect of $N_s$ is easy to understand: the larger the density of surface groups, the higher the pH sensitivity because more groups are able to participate in the protonation-deprotonation reaction. $K_a$ and $K_b$ reflect the position of two surface transition points on the pH scale called pKs. pKs denotes the transition of surface charge from positive to neutral, and pKb denotes the transition from neutral to negative. If the ΔpK is small, the surface transitions quickly from positive to negative without encountering an extended neutral region, imparting it with a high pH sensitivity. These relationships can be seen in Figure 21, where we simulate a hypothetical situation with $C_{dl} = 0.16$ F/m$^2$ [51], temperature = 298 K, and varied $N_s$ and ΔpK.

![Figure 21](image)

**Figure 21.** Simulated results of pH sensitivity ($\frac{d\psi}{dpH}$) dependence on (a) surface site density ($N_s$) and (b) separation between acid and base constants (ΔpK).

$N_s$ and ΔpK values have been reported for different oxides previously based on empirical fits. Table 2 lists the collected literature values for SiO$_2$ vs. Al$_2$O$_3$, two
commonly used dielectric materials in biosensing. We take the average of these reported values and plot SiO$_2$ vs. Al$_2$O$_3$ sensitivity in Figure 22, assuming symmetry around the point of zero charge. From this, one can see that SiO$_2$ is non-Nernstian over nearly all pH values. SiO$_2$ sensitivities reported in literature show a large range of values [44, 52-54], likely depending on the quality of the layer, the quality of the reference electrode, and the pH range of operation. The large ΔpK of SiO$_2$ leads to low pH sensitivity close to its point of zero charge, but near Nernstian sensitivities (around 58 mV/pH) have been reported experimentally within a small range around neutral pH [53]. Unmodified SiO$_2$ is also highly nonlinear in pH response. Efforts have been made to linearize its pH through surface modification. For example, self-assembled monolayers including aminopropyltriethoxysilane (APTES) and HMDS have been successfully used to linearize the pH response [55, 56]. Al$_2$O$_3$ on the other hand, has a small ΔpK, therefore exhibits linear and near-Nernstian behavior over a large pH range without any surface modification. High-k dielectrics other than Al$_2$O$_3$ have also been investigated in pH sensing, including HfO$_2$ [57, 58] and Ta$_2$O$_5$ [59], and have also shown sensitivity close to the Nernst limit.

<table>
<thead>
<tr>
<th>Material</th>
<th>$N_s$ (m$^{-2}$)</th>
<th>ΔpK</th>
<th>Point of zero charge</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$</td>
<td>5×10$^{18}$</td>
<td>8</td>
<td>2</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>5×10$^{18}$</td>
<td>6.4</td>
<td>2.1</td>
<td>[55]</td>
</tr>
<tr>
<td>Al$_2$O$_3$</td>
<td>8×10$^{18}$</td>
<td>4</td>
<td>8</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>8×10$^{18}$</td>
<td>1.5</td>
<td>7.9</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>8×10$^{18}$</td>
<td>0.4</td>
<td>7</td>
<td>[51]</td>
</tr>
</tbody>
</table>
Figure 22. Simulated pH sensitivity of SiO$_2$ and Al$_2$O$_3$.

5.2 Signal to Noise Ratio of Al$_2$O$_3$ vs. SiO$_2$

The important performance metric affecting limit of detection and resolution is signal to noise ratio (SNR). Therefore, higher sensitivity alone is not an adequate enough reason for the selection of one sensing dielectric over another. To look at the noise performance of different dielectrics, one must first revisit the theory.

Low frequency noise of FET devices is dominated by flicker noise, also known as $1/f$ noise, which takes on the general form $\frac{S_f}{I_{DS}} \propto \frac{1}{f}$. $S_f$ is the drain current noise power spectral density, and $f$ is frequency. The root mean square (RMS) current noise amplitude $\tilde{\alpha}$ is obtained by integrating $S_f$ over the entire measurement bandwidth and taking the square root of this result:
\[
\delta i = \sqrt{\int_{f_1}^{f_2} S_i \, df} = \sqrt{\int_{f_1}^{f_2} S_i \left( f = 1 \text{Hz} \right) \, df} = \sqrt{\ln \left( \frac{f_2}{f_1} \right) \times S_i \left( f = 1 \text{Hz} \right)}
\]

where \( f_2 \) and \( f_1 \) are the largest and smallest frequencies sampled in the measurement. The change in signal is measured as a change in current \( \Delta I_{DS} \), which is the change in surface potential scaled by the device transconductance. The SNR of a measurement is thus defined as [61, 62]:

\[
\text{SNR} = \frac{\Delta I_{DS}}{\delta i} = \frac{g_m \times \Delta \psi_0}{\sqrt{\ln \left( \frac{f_2}{f_1} \right) \times S_i \left( f = 1 \text{Hz} \right)}}
\]

(19)

\( S_i \) can be related to the power spectral density due to gate voltage fluctuations, \( S_{VG} \), by

\[
S_i = S_{VG} \times g_m^2
\]

(20)

Although traditionally in biological sensing, limit of detection is defined where \( \text{SNR} \geq 3 \), here, the smallest detectable surface potential for which \( \text{SNR} = 1 \) is also relevant, because it sets the limit on the smallest detectable signal from the device perspective. In that case, the voltage resolution (\( \Delta V_{\text{min}} \)) is then given by:

\[
\Delta V_{\text{min}} = \sqrt{\ln \left( \frac{f_2}{f_1} \right) \times S_{VG}}
\]

(21)

The SNR and \( \Delta V_{\text{min}} \) of a real sensing experiment is weakly dependent on the bandwidth of measurement through the \( \sqrt{\ln \left( \frac{f_2}{f_1} \right)} \) term. This term typically ranges from 2.5 to 4 depending on the actual experiment. Going forward in analysis, in order to compare SNR and resolution from different devices, we set the bandwidth term equal to 1, and express
the signal to noise ratio metric as per volt change in $\psi_0$. To distinguish this comparison metric from the terms we just introduced, we add a subscript “comp” to denote the comparison metrics:

$$\text{SNR}_{\text{comp}} = \frac{1}{\sqrt{S_{VG}}}$$

(22)

$$\Delta V_{\text{min, comp}} = \frac{1}{\text{SNR}_{\text{comp}}} = \sqrt{S_{VG}}$$

(23)

5.2.1 Signal to Noise Ratio of Yale Nanoribbons

Using our nanoribbon devices, we were able to compare signal to noise ratios of different devices with the same fabrication procedure and size, but different dielectric material. All noise measurements on nanoribbons are taken using the single device setup (see Section 4.4.1). The bandpass filter removes the DC component of the current signal and records only the AC fluctuations. The time domain noise signal is converted into a frequency domain signal by Fourier transform, using Matlab’s `pwelch` method [63]. Briefly, the program splits the signal into smaller overlapping segments, a fast Fourier transform (FFT) is performed on each segment, then averaged to obtain the noise power spectrum of the entire data set. Figure 23 presents the SNR$_{\text{comp}}$ values for SiO$_2$ and Al$_2$O$_3$ nanoribbon devices at different gating voltages. These devices were fabricated in parallel runs, and have dimensions of 10 µm × 1 µm. It is interesting to note that the range of maximum SNR$_{\text{comp}}$ have different trends for the two sets of devices, potentially due to the different material systems used. Studies that show maximum SNR in different regimes of FET sensor operation have been presented in the past [62, 64, 65].
Figure 23. SNR_{comp} as a function of gate voltage for (a) Al_{2}O_{3} and (b) SiO_{2} nanoribbons fabricated at Yale measured on the single channel setup.

Based on Equations 22-23, we can calculate various metrics of comparison from measured noise. Apart from SNR_{comp}, we can also determine voltage resolution ($\Delta V_{\text{min,comp}}$), charge resolution ($\Delta \sigma_{\text{min,comp}}$), and pH resolution ($\Delta \text{pH}_{\text{min,comp}}$). We expect that an increase in dangling bonds at the interface between silicon and dielectric would likely result in more interface traps and therefore higher noise [66]. In the case of SiO_{2}, the number of dangling bonds is significantly reduced via oxidation as they react with oxygen atoms to form the dielectric. However, in the case of deposited high-k dielectrics like Al_{2}O_{3}, the reduction of dangling bonds is achieved through high temperature annealing post-deposition, which is far less effective at reducing trap density. Although the Al_{2}O_{3}-Si interface is lower quality compared to the SiO_{2}-Si interface, SNR_{comp} is only slightly larger and $\Delta V_{\text{min,comp}}$ slightly smaller for SiO_{2} devices due to the higher capacitance of Al_{2}O_{3} compared to SiO_{2}. 

Given the charge sharing model previously described (Figure 6), where a change in surface charge density ($\Delta \sigma_0$) is screened by a combination of charges in the double layer and in the nanoFET, we arrive at $\Delta \psi_0 = \frac{\Delta \sigma_0}{C_{dl} + C_{nanoFET}}$, where $C_{nanoFET}$ is the nanoFET capacitance. The charge resolution $\Delta \sigma_{\text{min, comp}}$ is then given by $\left( C_{dl} + C_{nanoFET} \right) \Delta V_{\text{min, comp}}$. We assume that $C_{dl}$ does not change with the underlying dielectric (~0.16 F/m$^2$), and that $C_{nanoFET} \approx C_{ox}$ in accumulation (1.73×10$^{-3}$ F/m$^2$ for SiO$_2$; 3.98×10$^{-3}$ F/m$^2$ for Al$_2$O$_3$). Based on the calculation, it is clear that due to the slightly inferior (larger) $\Delta V_{\text{min, comp}}$ of Al$_2$O$_3$, it also exhibits slightly worse (larger) charge resolution. However, one can see that since the pH sensitivity of Al$_2$O$_3$ is more than double that of SiO$_2$ (Figure 24a&b), it more than compensates for the larger $\Delta V_{\text{min, comp}}$ of Al$_2$O$_3$, and as a result, the pH resolution of Al$_2$O$_3$ is much better than that of SiO$_2$. In other words, although Al$_2$O$_3$ is not able to detect as small of a voltage change due to its high defect density, it is able to detect a smaller pH change because each unit of pH change elicits a larger voltage response from the material.

Table 3. Measured average peak SNR$_{\text{comp}}$ for SiO$_2$ and Al$_2$O$_3$ nanoribbons and calculations of limits of detection ($\Delta pH_{\text{min, comp}}$ is calculated assuming 25 mV/pH for SiO$_2$ and 58 mV/pH for Al$_2$O$_3$)

<table>
<thead>
<tr>
<th>Material</th>
<th>Average peak SNR$_{\text{comp}}$ (V$^{-1}$)</th>
<th>$\Delta V_{\text{min, comp}}$ (V)</th>
<th>$\Delta \sigma_{\text{min, comp}}$ (charges/µm$^2$)</th>
<th>$\Delta pH_{\text{min, comp}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$</td>
<td>$(2.14\pm0.25)\times10^4$</td>
<td>$(4.74\pm0.60)\times10^{-5}$</td>
<td>48.51±6.17</td>
<td>$(1.90\pm0.24)\times10^{-3}$</td>
</tr>
<tr>
<td>Al$_2$O$_3$</td>
<td>$(1.84\pm0.56)\times10^4$</td>
<td>$(6.04\pm2.61)\times10^{-5}$</td>
<td>61.82±26.74</td>
<td>$(1.04\pm0.45)\times10^{-3}$</td>
</tr>
</tbody>
</table>
This difference in pH resolution can be seen in a pH sensing comparison by alternatingly flowing buffers narrowly separated in pH, as in Figure 24c&d. Al₂O₃ clearly exhibits a better resolution than SiO₂. The inferior experimental resolution compared to calculated values in Table 3 is partially due to the bandwidth factor in Equation 19 being larger than 1 in real sensing experiments.

In summary, motivated by the higher pH sensitivity and better resolution (based on noise considerations) of Al₂O₃-coated nanoFETs, we decided to pursue Al₂O₃ devices for our pH measurements. The enzyme-substrate detection data presented in this thesis (Section 7) are obtained using Al₂O₃-passivated nanoribbons. This also indicates that one should keep in mind the eventual application of the device when trying to design optimal sensors. If the application is one of direct sensing, where the voltage response is approximately the same for analyte binding on different material surfaces, SiO₂ may be the material of choice due to its better charge resolution.
Figure 24. Wide range pH sensing data from (a) Al₂O₃ and (b) SiO₂ nanoribbons. The y-axis is inverted for ease of visualization. Narrow range pH sensing data from (c) Al₂O₃ and (d) SiO₂ nanoribbons.

5.2.2 Signal to Noise Ratio of QuantuMDx Nanowires

During the recent years, we received state-of-the-art-fabricated Al₂O₃-coated nanowire FET devices from a collaborating company, QuantuMDx. These devices have excellent noise performance due to the higher standard cleanroom processing, and due to the presence of the interfacial SiO₂ layer (See Section 4.3). These devices also have very high uniformity across the chip, which allowed us to use the 32-channel portable PCB for
sensing under a global gating scheme (described in Section 4.4.3). We measured the noise performance of 16 devices using the 32-channel setup (Figure 25).

![Figure 25. SNR\textsubscript{comp} as a function of gate voltage for QuantuMDx Al\textsubscript{2}O\textsubscript{3}-coated nanowire measured on 32 channel setup.](image)

As with the nanoribbons, we measured pH sensitivity of the QuantuMDx nanowires by flowing buffers across the surface (Figure 26), from which we can calculate 

\[ \Delta pH_{\text{min}, \text{comp}} \]
Figure 26. Wide range pH sensing data from Al₂O₃ nanowires. The pH-sensitivity was averaged from 9 devices. The y-axis is inverted for ease of visualization.

The comparisons between Al₂O₃ nanoFET devices from Yale and QuantuMDx are summarized in Table 4. One can see that even with the smaller dimensions of the nanowires and the inevitably higher noise floor of the PCB, we see that the SNR\textsubscript{comp} of nanowires measured using this setup compares favorably to that of the nanoribbons measured on the single-channel low noise setup. In addition, these devices had much better uniformity across each chip compared to the Yale-fabricated devices. Therefore, the immuno-PCR and immuno-LAMP experiments were performed using this platform (presented in Sections 7 and 8).

<table>
<thead>
<tr>
<th>Device</th>
<th>SNR\textsubscript{comp} (V\textsuperscript{-1})</th>
<th>(\Delta V)\textsubscript{min,comp} (V)</th>
<th>(\Delta \sigma)\textsubscript{min,comp} (charges/µm\textsuperscript{2})</th>
<th>(\Delta \text{pH})\textsubscript{min,comp}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoribbon</td>
<td>(1.84±0.56)×10\textsuperscript{4}</td>
<td>(6.04±2.61)×10\textsuperscript{-3}</td>
<td>61.82±26.74</td>
<td>(1.04±0.45)×10\textsuperscript{-1}</td>
</tr>
<tr>
<td>Nanowire</td>
<td>(1.93±0.21)×10\textsuperscript{4}</td>
<td>(5.23±0.59)×10\textsuperscript{-3}</td>
<td>53.56±6.01</td>
<td>(9.02±1.01)×10\textsuperscript{-4}</td>
</tr>
</tbody>
</table>
5.3 Other Benefits of Al₂O₃

In addition to the aforementioned advantages of Al₂O₃, it also allows for effective and robust isolation of device channel and solution, preventing leakage current from increasing device noise. Furthermore, the dielectric is a high density material with a low hydration rate, which prevents device drift and dielectric breakdown due to ion incorporation [67]. As shown in Table 2, Al₂O₃ is feebly charged at neutral pH, therefore reducing fouling due to nonspecific adsorption and thus allowing device reuse. The latter is particularly important for assays where multiple reactions are to be measured using the same device.

5.4 Device Scaling

Although the scaling effect is not apparent between the nanoribbon and nanowire devices we used due to the different fabrication processes involved, we feel compelled to discuss this topic, as it will be important moving forward in the field. A large body of literature is available highlight all the applications that have been demonstrated using devices with a large range of dimensions, however, many of these works are presented without background information regarding why a device of that particular size was chosen. Therefore, before discussing applications, an important issue is device scaling – i.e., what is the optimal size of the nanoFET sensor which will enable the best performance?

The tremendous research interest in the nanowire field has been based on the premise that nanowire devices offer excellent current sensitivity due to their large surface
area-to-volume ratio, with sensitivity commonly defined as \( \frac{\Delta I_{DS}}{I_{DS}} \) (which, we must note, is different from the true definition of sensitivity – see Box 1). With this being the metric, smaller devices are clearly superior, and significant research effort was devoted to scaling to small dimensions [32], approaching 1D. In addition, a few other advantages of downscaling dimension have come to light – in addition to a small improvement in the electrostatics, the geometry of thinner wires may also offer reduced detection time due to an improvement in the analyte diffusion kinetics [68]. However, in a real biological experiment, the limit of detection (when measuring low concentration samples) and resolution (when measuring pH changes) are more critical parameters, and the metric of sensitivity provided above offers no information in these regards. As discussed earlier in this section, the characteristic that determines the smallest resolvable \( \Delta I_{DS} \) is the intrinsic current noise of the sensor. Therefore the metric of SNR becomes more relevant in comparing the detection limit of the sensors of different sizes, with higher SNR corresponding to lower detection limits [61].

When using SNR as the metric of interest, one can see that smaller devices may not be better for every application. Again, the SNR of a measurement can be described as in Equation 19, repeated below.

\[
\text{SNR} = \frac{\Delta I}{\partial t} = \frac{g_m \times \Delta \psi_0}{\sqrt{\ln \left( \frac{f_2}{f_1} \right) \times \sqrt{S_f(f = 1 \text{ Hz})}}}
\]
where $\Delta \psi_0 = \frac{\Delta \sigma_0}{(C_{ox} + C_{dl})} = \frac{\Delta Q_0}{A(C_{ox} + C_{dl})}$, with $\Delta Q_0$ being the total change in surface charge, and $A$ being the device surface area.

$S_i$ in Equation 19 can be expanded further by considering the number fluctuation model [69, 70]:

$$S_i = g_m^2 \times S_{vFB} = \frac{g_m^2 (\lambda k T q^2 N_0)}{A \times C_{ox} \times f}$$  \hspace{1cm} (24)$$

where $S_{vFB}$ is the flatband voltage noise power density, $\lambda$ is the characteristic tunneling distance in the material, and $N_0$ is the interface trap density. Thus we can see that SNR can be represented in the following form:

$$\text{SNR} \propto \frac{\text{constant} \times \Delta Q_0}{\sqrt{A}}$$  \hspace{1cm} (25)$$

From Equation 25 one can see that if $\Delta Q_0$ scales with area, SNR is higher for devices with larger surface area; conversely, if $\Delta Q_0$ is constant, SNR is higher for devices with smaller area. For common diagnostic applications for concentration determination, the probability of analyte binding scales linearly with available surface area, therefore nanoribbons and nanoplates may be more desirable. On the other hand, in the case of single-molecule kinetic studies, where only one single receptor is engineered on each device such that only one analyte is allowed to bind at any time, smaller nanowire devices may be more desirable. However, the calculations do not take into account any quantum confinement induced volume inversion effects, which may play into effect at very small wire widths of 5-10 nm to decrease device noise [71].
For pH related applications, all exposed dielectric surfaces will participate in the protonation-deprotonation reaction, therefore, the obtained signal scales with device area. Indeed, noise studies of pH measurement performance from several groups have recently demonstrated the advantage of larger area devices in this aspect [34, 72]. Therefore, for the devices we fabricated in-house, we chose large dimension devices on the order of 10 to 20 µm by 1 to 50 µm, which not only provided a great SNR, but also simplified the fabrication process significantly compared to smaller devices. Of course, these conclusions assume constant $N_0$ among the comparative devices (however, different sized devices often require different fabrication techniques, which can significantly alter the noise level). We can see from the comparison between the Yale nanoribbon and QuantuMDx nanowire FETs (Table 4) that the processing quality and the presence of intermediate SiO$_2$ layer can also have a major influence on the performance of the device. Nevertheless, device dimensional design guided by maximizing SNR is a useful approach to obtaining optimized FET sensors for different applications.

In the biologically relevant pH-sensing work presented in Sections 6-8, different sized Al$_2$O$_3$-coated devices were used, depending on availability. The enzyme-substrate interaction monitoring presented in Section 6 was performed using our nanoribbon and nanoplate devices. When the Al$_2$O$_3$ nanowire devices from QuantuMDx were provided to us, we switched to these devices for the work on immune-PCR and immune-LAMP, presented in Sections 7 and 8.
6.1 Background

The need for high-throughput, rapid, and sensitive assessment of enzyme and substrate interaction is of central importance in the fields of clinical diagnosis and treatment, as well as for the discovery of cellular and organismal metabolic regulators. The catalytic activity for enzyme-substrate interactions is an informative measure, as activity within a biological sample is indicative of the functional output of the enzyme and can be more easily related to cellular or organismal phenotype. Current methods of activity detection mostly include colorimetric, fluorometric, chromatographic, and radioactive assays, which either lack sensitivity, or require bulky and expensive equipment (e.g., spectrophotometers) and tedious procedures [73]. Many current methods
require the use of a labelled substrate, which is not only difficult to synthesize, but may alter its interaction with the enzyme. Other methods based on electrochemical assays have also been reported, but are typically limited to redox reactions [74-76].

Many enzymatic reactions release or consume acidic or basic products, and thus alter the pH of their environments [77], suggesting FET-based approaches and a simple, cost-effective method for the detection of these enzymatic reactions. The idea of the enzymatically coupled ion-sensitive field effect transistor (ENFET) was postulated in 1976 [78], and realized in 1979 to detect urea [79]. Since then, various biologically relevant substrates have been detected, such as urea [80-82], creatinine [83, 84], glucose [85-87], penicillin [88, 89], and acetylcholine [90, 91].

Prior work focused on the immobilization of enzymes on the surface of the ENFETs, where substrate addition induced a pH change [88, 92]. This method cannot be used for enzyme activity quantification, as substrate immobilization is difficult, and may render the substrate unrecognizable by the enzyme. In addition, enzyme immobilization presents several significant drawbacks that have hindered the adoption of ENFETs: (1) immobilization may alter the activity of the enzyme compared to its natural environment [93], which precludes meaningful extraction of kinetic constants; (2) the requirement of device functionalization does not allow for repeated use, introducing device-to-device variability in measurements and significantly increasing cost; (3) functionalizations tend to be unstable in solution, with degradation leading to device instabilities over time; and (4) repeatable functionalization of membranes with proteins remains a challenging problem, which leads to issues with reproducibility.
We show that the drawbacks enumerated above can be overcome by combining the enzyme and substrate directly in a small reservoir encasing unfunctionalized nanoribbons. These nanoribbons are fabricated in the Yale Cleanroom (as described in Section 4.1), and the theoretical reason for using larger area devices like ribbons is motivated in Section 5.5.

We demonstrate the versatility of this approach through the detection of both substrate and enzyme in physiological buffers, and the characterization of kinetic constants. Specifically, we show detection of urea in PBS with exceptional linearity over the normal physiological range, high sensitivity detection of penicillinase in PBS and urine, and accurate analysis of the enzyme kinetics of the acetylcholinesterase-acetylcholine reaction, demonstrating that this approach can be generalized to a high-throughput platform for the detection of enzyme substrate interactions across many reaction classes.

Post-fabrication and post-dicing, individual dies are wire bonded to 28 pin DIP packages. A fluid reservoir is created by gluing a polypropylene tube on the surface of the chip to hold solution over the device (as described in Section 4.5.1). The exposed wirebonds are sealed with epoxy (Devcon) to protect them from solution.

The devices we used varied in length between 10 and 20 µm, and varied in width between 1 and 32 µm. Figure 27a shows a 3D optical profile image (Zeta-20; Zeta Instruments) of a 20 µm long and 2 µm wide ribbon. The top down process allows us to precisely and consistently define the dimensions and location of the nanoribbons, which allows for high device yield and repeatable electrical characteristics. The single channel
measurement system is used to interrogate individual devices (as described in Section 4.4.1). The experimental setup involves the application of a drain voltage \( V_{DS} \), typically 0.02 to 0.1 V and gate voltage \( V_{GS} \), typically -0.2 to -0.5 V to set the operating point (Figure 27b). The gate voltage is applied via a pseudoreference gate electrode made from an Ag/AgCl wire immersed in the solution contacting the device (see Section 4.5.2). The drain current is filtered, amplified, and converted into a voltage, which is read by a DAQ card in real time. A typical current vs. gate voltage device characteristic is shown in Figure 27c.

In order to determine pH sensitivity of the nanoribbons used for this experiment, the devices were exposed to standard pH calibration solutions. Our devices are \( p \)-type, therefore an increase in pH corresponds to an increase in \( I_{DS} \), as deprotonation of siloxyl groups leads to increased negative charges on the surface. We measured the pH response of 5 devices randomly sampled across the wafer, and obtained a pH sensitivity of 55.44 ± 2.94 mV/pH, close to the limit of 59.1 mV/pH at 25°C. In order to suppress non-specific adsorption of enzymes on charged surfaces (such as exposed SU-8), the entire solution reservoir was incubated with 3% bovine serum albumin (BSA; Fisher) in PBS (pH = 7.4; Gibco) for one hour the first time each bonded die is used. The pH response was measured again after BSA treatment, and a similar pH response of 53.5 ± 3.03 mV/pH (7 devices randomly sampled) was obtained, indicating little non-specific adsorption of proteins on the near-neutral \( \text{Al}_2\text{O}_3 \) surface. A representative pH response plot is shown in Figure 27d (53.3 mV/pH, \( R^2 = 0.996 \)), showing both high sensitivity and linearity after blocking.
Figure 27. Typical nanoribbon device characteristics. (a) optical image of a nanoribbon device ($L = 20 \, \mu m$, $W = 2 \, \mu m$) with a $2 \, \mu m$ deep via in SU-8. (b) schematic of device operation. The pH change due to enzyme (green) and substrate (yellow) interaction is measured. (c) typical current vs. gate voltage characteristic of a nanoribbon device ($L = 10 \, \mu m$, $W = 1 \, \mu m$, subthreshold swing $\sim 80 \, mV/dec$). (d) pH sensitivity of a nanoribbon device after BSA blocking.

In our experiments, enzyme and substrate are combined in solution and allowed to react (Figure 28a). The enzyme (green) catalyzes the conversion of substrates (yellow) into products (pink), which can alter the pH of its environment in the process (Figure 28b). The change in pH can be measured by the nanoribbon in solution in real time, and
is reflected in the change in $I_{DS}$ through the device. The signal saturates to a stable value when the pH of the system shifts out of the optimal operation range and inactivates the enzyme, or the reaction goes to completion and all of the available substrates are converted into products (shown in Figure 28c).

![Figure 28](image.png)

**Figure 28.** Overview of nanoribbon detection of enzyme (green) and substrate (yellow) interactions via pH change. (a) beginning of reaction. (b) enzymatic conversion of substrates to products with passing time. (c) reaction is completed when all substrates have been converted to products.

### 6.2 Substrate Detection

We use the urea-urease reaction as a model system to demonstrate the ability of silicon nanoribbons to carry out substrate detection. Urea is an important compound in metabolic processes and its concentration in physiological solutions, such as blood or urine, is an important diagnostic measure of renal, liver, heart, and other diseases [94-96]. The normal level of urea in human serum is 1.9-7.7 mM [97], and elevated levels are indicative of renal damage.
Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide (Figure 29a). The ammonia released into solution is spontaneously protonated to ammonium, while the CO$_2$ is changed to bicarbonate. Since twice as much NH$_3$ is released compared to CO$_2$, the pH increases as the reaction proceeds.

The detection of urea is demonstrated using different concentrations of urea (Sigma) diluted into PBS. 100 µL of the urea solution was added to the sensing reservoir and a stable baseline was obtained. 50 µL of a high concentration solution (4.5 mg/mL) of urease from *Canavalia ensiformis* (EC 3.5.1.5; Sigma) was subsequently added by pipetting, and the response was monitored. Time = 0 was defined after recovery to baseline after a brief (~few seconds) transient due to fluid injection. The initial current at time = 0 ($I_{DS0}$) was subtracted from the measured current for each concentration ($I_{DS}$), and the difference was divided by the transconductance in the linear regime ($g_m$) to calculate the change in surface potential ($\Delta \psi$), as in Equation 5. The results are plotted in Figure 29b. The small peak observed near the start of the reaction is likely a result of the faster ammonia protonation compared to the bicarbonate formation. As the reaction proceeds, the signal approaches a stable value. The difference between this limiting value and the control value can be related to the final pH change in solution (Figure 29c) using

$$\Delta \text{pH} = \frac{I_{DS} - I_{DS0}}{g_m \times \gamma}$$

(26)

where $\gamma$ is the pH sensitivity of the device (53.5 mV/pH). Despite the strong buffering capacity of the PBS, urea detection can be achieved with a starting urea concentration as low as 167 µM ($\Delta \text{pH} = 0.023$). This limit can be further decreased by adding a smaller
volume of concentrated enzyme solution to reduce signal dilution. The normal range for blood urea is colored cyan in Figure 29c.

Figure 29. Detection of urea using nanoribbon pH sensors. a) hydrolysis of urea, catalyzed by urease, producing ammonia and carbon dioxide as products (basic groups are shown in blue, acidic groups are shown in red); b) potential signal from urea-urease reaction vs. time, with different urea concentrations. The y-axis is inverted for ease of visualization. c) same data in b) converted into a pH change using Equation 26, plotted on a semilog scale. Inset shows a zoom in of the physiologically-normal range (highlight in cyan), plotted on a linear scale.

Unlike the methods requiring functionalization, this method is insensitive to variations in enzyme concentration, and small variations in enzyme activity due to changes in pH, as the metric of interest is the final pH change in comparison to the control. Over the expected blood urea concentration range, the signal is linear (R=0.986; Figure 29c, inset). At extremely high urea concentrations, the signal tends towards...
saturation, most likely because either the pH or NH₄⁺ concentration has changed so much that the activity of the enzyme is significantly inhibited [98, 99], and therefore substrates were not exhausted. In applications where a larger dynamic range is desired, an additional feedback unit can be used to readjust the pH of the solution back to the optimal point throughout the reaction [100].

6.3 Enzyme Activity Detection

In addition to substrate detection, another appealing feature of the approach is the ability to carry out enzyme activity quantification. We show that by using the nanoribbon as a pH sensor without functionalization, we can easily and reliably measure enzyme activity to very low levels. To demonstrate this, we use the nanoribbons to detect penicillinase, an enzyme that hydrolyzes penicillin G. Despite the rapid development of penicillin resistance in bacteria with the introduction of penicillin in the 1940s [101], penicillin remains an important antibiotic used in the medical industry today, as many strains of bacteria are still susceptible. Therefore, it is critical to be able to measure penicillin resistance, which is frequently attributed to the presence of penicillinase [101]. Current methods of penicillinase detection involve the use of a penicillin disk [102], which lacks sensitivity. The hydrolysis of penicillin G releases a proton and lowers the pH of the solution as shown in Figure 30a. By mixing the enzyme with a high concentration of penicillin, we can measure the rate of pH decrease, and thus deduce the enzyme concentration.

We varied the concentration of penicillinase from Bacillus cereus (EC 3.5.2.6; Sigma) from 0 to 20 units/mL in PBS (a unit of enzyme is defined as the amount of
enzyme required to catalyze 1 µmol of substrate at optimal pH and temperature), and added 100 µL of the enzyme solution into the cup to achieve a steady baseline. 50 µL of a high concentration (12.8 mg/mL) of penicillin G (Sigma) was then added and mixed by pipetting. \( \Delta I_d \) was monitored for 30 minutes from the moment the mixing was complete (Figure 30b), and subsequently converted to the equivalent pH change rate using Equation 26 (Figure 30d, red). Each data point was repeated 5 times, the penicillinase-free control was repeated 3 times, and the mean control rate was subtracted from the rate measured for each concentration. The standard errors of the mean are shown for each PBS data point in Figure 30d. After only a few minutes of reaction time, the detection limit can reach as low as 0.2 units/mL, and within 30 minutes, the detection limit can reach 0.02 units/mL, with the potential of becoming lower with even longer incubation time. Al₂O₃ is known to have low drift (< 1 mV/hr, equivalent to <0.02 pH units/hr), allowing long-term measurements to be taken [67]. For this sample of penicillinase, 0.02 units/mL is equivalent to an enzyme concentration of 300 pM.

Penicillin-resistant urinary tract infections (UTIs) are a common condition [103], and blood sepsis patients can show bacteria in urine [104]. Therefore, we addressed the question of whether we can achieve effective penicillinase detection in urine. A urine sample (pH 7-7.5) was collected, filtered through a 0.22 µm pore syringe filter, and kept at 4 °C until use. Experiments were conducted within 48 hours of sample collection. We conducted the same penicillinase sensing experiment in urine as we did for PBS (Figure 30c). We found that the initial rate of signal change in urine was considerably slower than that of PBS, and we had to monitor the reaction over a longer time period in order to maintain the same detection limit. As a result, we were able to detect 0.02 units/mL of
penicillinase in urine in just under 2 hours. The initial rate of change in the pH as a function of penicillinase activity is shown in Figure 30d (blue). We found that the ratio between the rates of pH change in PBS and urine is $2.77 \pm 0.76$. It is also worth noting that as the signal saturates due to substrate depletion, the saturated signal for PBS is higher than that of urine by approximately the same ratio, despite the substrate concentration being the same in both sets of experiments. This indicates that the difference between urine and PBS is mainly a result of the higher buffering capacity of urine, caused by the presence of phosphate, ammonia, various organic solutes, and proteins. To test this, we measured the buffering capacity of these two sample solutions by generating a pH titration curve. We titrated PBS and urine with HCl over the nanoribbons and measured the pH response. 2 µL of HCl solution of different concentrations was added to 150 µL of the sample buffer, and the pH response was extracted and plotted against the final change in proton concentration (Figure 30e). Due to buffering, small changes in proton concentrations near the buffers’ pK lead to a linear change in pH. The data within 1 unit of pH change from original was fit with a straight line, and the slope is extracted as the buffering capacity. It was found that the buffering capacity of urine close to the original pH of the solution was approximately 3.4 times that of PBS (Figure 30e), consistent with the difference we observed between the rates and signals of the PBS versus urine measurements (Figure 30d). This is an important calibration method, as an issue with pH-based detection is the variability of buffering capacity between different biological samples, and thus the difficulty of generalizing the method of pH detection across all types of solutions. Thus, this challenge can be easily overcome by generating a titration curve in the buffer of interest prior to running the
enzymatic reaction, and will allow for the estimation of enzyme activity in complex solutions or solutions differing in buffer capacity.
Figure 30. Detection of penicillinase enzyme activity using nanoribbon sensors. (a) reaction between penicillin and water, catalyzed by penicillinase, producing penicilloic acid as product (acidic groups are shown in red). (b) potential signal from penicillin-penicillinase reaction in PBS vs. time, with different penicillinase activities. (c) potential signal from pencillin-penicillinase reaction in urine vs. time, with different penicillinase activities. (d) titration data of HCl in PBS (red) and urine (blue). Linear fits to the data show the ratio of the buffering capacities (3.4) of the two samples; e) initial response rates from b) and c) converted into a pH change rate using Equation 26.
6.4 Kinetic Constant Extraction

Nanoribbon sensors have the additional advantage of real time signal transduction, which permits the extraction of kinetic constants of the system [4]. To demonstrate this application for enzyme-substrate pairs, we use the system of acetylcholine and acetylcholinesterase. Acetylcholinesterase controls the half-life of acetylcholine, a crucial neurotransmitter. The ability to probe the interaction between acetylcholine and its esterase may provide valuable insight into the termination of synaptic transmission. From the reaction of acetylcholine and acetylcholinesterase (Figure 31a), one can see how this pH detection method can be generalized to probe all reactions catalyzed by esterases, as the products will always yield a carboxylic acid group and an alcohol group. The carboxylic acid deprotonates at neutral pH, thus increasing the acidity of the solution. One of the best-known models of enzyme kinetics is based on the Michaelis-Menten equation:

\[ v = V_{\text{max}} \frac{[S]}{K_m + [S]} \]  

(27)

Where \( v \) is the initial rate of product generation, \( V_{\text{max}} \) is the maximum rate of product generation at saturating substrate concentration, \([S]\) is the substrate concentration, and \( K_m \) is the Michaelis-Menten constant, equivalent to \([S]\) at which \( v \) reaches \( \frac{1}{2} V_{\text{max}} \). \( K_m \) is an important kinetic value describing the affinity between the enzyme-substrate pair, and can be extracted from initial reaction rates as a function of substrate concentration.
To monitor the kinetics of the reactions and determine $K_m$, we carried out the reaction in water, so that initial reaction velocities can be easily determined and the pH changes will remain unbuffered. We compared the results of our method against spectrophotometric fluorescence detection, which is a commonly accepted method of extracting $K_m$. A series of experiments was conducted using each method at room temperature, where the enzyme concentration was held constant (1.28 units/mL) and the substrate concentration was varied. The same batch of acetylcholinesterase (EC 3.1.1.7; Life Technologies) and acetylcholine (Life Technologies) were used for each experiment. In the nanoribbon assay, acetylcholine and acetylcholinesterase from *Electrophorus electricus* were mixed in molecular grade water (supplemented with 150 mM NaCl and pH adjusted to 8; Sigma) in a fluid reservoir over the nanoribbon. The pH change was monitored over time. In the fluorescence assay (Life Technologies), the same acetylcholine and acetylcholinesterase were mixed together in a 96 well plate in 50 mM Tris-HCl, pH = 8.0. The choline product from the reaction was oxidized by choline oxidase to produce $\text{H}_2\text{O}_2$, which in the presence of horseradish peroxidase reacts with Amplex Red to yield resorufin, a highly fluorescent molecule. Fluorescence was measured over time using the SpectraMax Paradigm microplate reader (Molecular Devices) at an excitation of 555 nm and emission of 595 nm.

The nanoribbon signal was converted to $\Delta$pH vs. time using Equation 26, then to proton concentration $[\text{H}^+]$ vs. time using

$$[\text{H}^+] = 10^{-8+\Delta\text{pH}}$$

(28)
with time = 0 being the moment the transients from mixing have subsided (Figure 31b). The initial 1-2 minutes exhibited significant nonlinearity due to slight buffering from the proteins in the system, so initial reaction rates were extracted from the slopes in the linear regime. In the fluorescence experiment, the initial slope was extracted using SoftMax Pro Software (Molecular Devices) in the linear range of the assay. We fit the Michaelis-Menten equation to the initial reaction rate versus substrate concentration data from both methods, and we show both on the same graph (Figure 31c). $K_m$ values of $(3.33 \pm 0.89) \times 10^{-4}$ M and $(1.79 \pm 0.19) \times 10^{-4}$ M were obtained on the nanoribbon and spectrophotometer, respectively (Table 5). The reported value of $K_m$ for acetylcholinesterase from *Electrophorus electricus* reacting with acetylcholine is $7.39 \times 10^{-5}$ M, using a matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) based assay [105]. The $K_m$ value obtained on the nanoribbon is very similar to that obtained using fluorescence, and both values are in reasonable agreement with previously reported value in literature considering sample variations, showing that nanoribbons are a viable method of probing enzyme-substrate affinity.
Figure 31. Analysis of acetylcholinesterase enzyme kinetics using nanoribbon sensors. (a) Reaction between acetylcholine and water, catalyzed by acetylcholinesterase, producing acetic acid and choline as products (acidic groups are shown in red). (b) Nanoribbon signal from acetylcholine-acetylcholinesterase reaction converted into its equivalent proton concentration over time. (c) Michaelis-Menten fits to nanoribbon and fluorescence data.

Table 5. Comparison of $K_m$ values obtained via different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>$K_m$ (M)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoribbon</td>
<td>$(3.33 \pm 0.89) \times 10^{-4}$</td>
<td>0.973</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$(1.79 \pm 0.19) \times 10^{-4}$</td>
<td>0.994</td>
</tr>
<tr>
<td>Literature (MALDI-FTMS [105])</td>
<td>$7.39 \times 10^{-5}$</td>
<td>0.995</td>
</tr>
</tbody>
</table>
6.5 Conclusion and Future Outlook

In conclusion, we have shown that properly designed electronic nanoFETs can be used as sensitive and reliable devices for measuring enzyme-substrate interactions. Substrate and enzyme concentration can be detected using pH change, even in highly buffered solutions. For substrate detection using urea as a model system, we have demonstrated a detection limit of less than 200 μM in PBS. We have also demonstrated an enzyme detection limit of 0.02 units/mL for the penicillin-penicillinase model system in PBS and urine, which we note is a general detection limit applicable to other enzymatic reactions that produce a proton. We also showed that enzyme kinetics can be analyzed to accurately determine the kinetic constant $K_m$, and we validated our technique against fluorescence spectroscopy. Our electronic method is readily generalized to many unrelated classes of substrates and enzymes. An additional advantage of our method of enzyme detection is that it does not require the use of high-affinity antibodies, which have limited availability for many enzymes. Our approach also allows the incorporation of on-chip control devices coated with a material with subdued pH response, such organosilanes [51] or polymers [106], so that differential measurements can be taken.

We believe that this method can be adapted to a simple, compact, inexpensive, and sensitive platform for high-throughput detection of different enzyme activities and substrate concentrations. The small size of our devices offer potential for future scaling to much smaller volumes as well as potential integration with other on-chip signal processing components. Applications of our method include, but are not limited to, high-throughput in vitro and in vivo mutant and RNA-interference screening to identify novel
metabolic regulators; and comprehensive profiling of patient tissues and fluids for personalized disease diagnosis and therapeutic monitoring.

* This work is published in [5].
INDIRECT QUANTIFICATION OF IMMUNO-PCR

7.1 Background

Clinical monitoring of biomarkers for disease diagnosis, treatment, or forensic applications requires the identification and quantification of low levels of proteins in small volumes of complex biological samples, often as low as femtomolar concentrations [107-109]. NanoFETs have shown superb performance as low noise, low power, and high sensitivity biosensors, and nanoFETs fabricated using CMOS-compatible techniques are particularly desirable for their potential ease of integration on-chip with other electrical components (resistive heaters, temperature sensors, signal processing circuitry, etc.). Therefore, the development of highly sensitive and specific nanoFET-based protein detection strategies is of high interest. Most of the work on nanoFET detection of
proteins thus far has been performed in low ionic strength solutions \([7, 110, 111]\); however, detection in high ionic strength, complex physiological buffers such as blood serum remains challenging due to the reasons described in Section 3.1, and often require desalting methods which can alter protein stability \([10, 11, 15, 112]\). For these reasons, an indirect sandwich immunoassay that converts the presence of the target protein to an amplified pH response is highly desirable, with the added benefit of two antibodies for increased specificity. Due to the small size of protons, pH detection is immune to the limitations associated with direct protein sensing. NanoFETs have been previously used to detect pH changes due to cellular activation \([22]\), enzyme-substrate interactions \([5]\), and buffer exchange \([32, 58, 113]\). ELISAs have also been reported to detect proteins indirectly, using an enzyme (such as urease) to catalyze pH change \([24-26]\); however, the linear amplification rate of the enzyme limits the potential sensitivity of the system (Figure 32).

**Figure 32.** Schematic depiction of linearly amplifying vs. exponentially amplifying immunoassays. \(t\) is time, \([E]\) is the enzyme concentration, \([DNA_i]\) is the initial DNA concentration, and \(\alpha\) is the thermocycling rate (cycles per time). If we assume that the concentration of the analyte of interest is equivalent in both cases, and the same linker is used to attach the enzyme and the DNA to the detection antibody, then \([E] = [DNA_i]\).
Owing to the exponential nature of nucleic acid amplification, immunoassays can be made orders of magnitude more sensitive by replacing the conventionally used enzyme with a DNA probe [114-116], in a process called immuno-PCR (Figure 32, detailed step-wise procedure provided in Figure 37a). Despite its advantages, immuno-PCR has never been used to improve protein detection by nanoFET devices. In this work, we performed indirect sandwich immunoassay combined with downstream PCR, with signal transduction by nanoFET sensors. During DNA amplification, the attachment of each nucleotide to the nucleic acid strand results in the release of a proton, changing the pH of the solution (see Figure 33 for mechanism). Our ultrasensitive nanoFETs with superb pH resolution allow us to detect these minute changes and correlate them to the original protein analyte concentration. We are able to achieve highly sensitive protein detection in full serum, as demonstrated using a model cytokine interleukin-2 (IL-2), while retaining the aforementioned benefits of an electronic readout system. We show detection of IL-2 concentrations from <20 fM to >200 pM, surpassing both the linear nanoFET urease-based readout as well as the conventional ELISA on which the model is based.
Figure 33. Chemical reaction during nucleic acid synthesis. A proton is released for every incorporated nucleotide from the 3’ hydroxyl group (red) on the growing nucleotide chain. Because of a larger number of such reactions, longer DNA templates will produce a proportionally larger pH change per PCR cycle. Any nucleic acid amplification following this mechanism will result in pH decrease.

Our nanoFET measurement setup consists of a PCB that can measure up to 32 devices simultaneously (Figure 18, Figure 34a, described in Section 4.4.3). A microfluidic channel encapsulating the nanoFETs brings in solution sequentially for measurement. Solution withdrawal allows quick exchanges between samples, with each measurement requiring no more than a few microliters of solution. A change in solution pH elicits a change in the conductance of the nanoFETs, which is low-pass filtered and converted into a voltage ($V_{out}$). A multiplexer sweeps the channels being measured, and the resulting signal is fed into a portable DAQ card (USB-DAQ) connected to a computer. The PCB is powered by batteries and is fully portable, and has the potential for further miniaturization in the future.

The nanoFETs used for these experiments are Al$_2$O$_3$-coated nanowire devices supplied by QuantuMDx (see Section 4.3). A close-up of representative nanoFET devices
on a chip is shown in Figure 14a, and a scanning electron micrograph of a single nanowire is shown in Figure 14b. In a typical experiment, 8-16 devices were measured simultaneously to obtain an average reading of the pH signal. Figure 34b presents the gating behavior from 15 devices on one chip, showing excellent uniformity. Measurements were taken in 1x PBS with the solution gate voltage swept from 0V to -2V then back to 0V. Negligible hysteresis is observed in the nanoFET devices.

Figure 34. Measurement setup and device characteristics. (a) Schematic of portable sensing board for simultaneous device measurement. (b) Drain current and transconductance vs. gate voltage scans of 15 devices on one chip. (c) Signal responses to gate steps on a representative device. Changes in applied gate voltage are shown on top.
In our measurements, the surface potential signal ($\Delta \psi$) was extracted by taking the difference between the measured current value ($I_{DS}$) and the current value at time 0 ($I_{DS0}$), and dividing by the device transconductance ($g_m$), as in Equation 5, which normalizes the variations between devices. Device sensitivity was assessed by flowing buffers of pH 4, 6, and 8, sequentially over the nanoFETs, and was determined to be 59.4 ± 2.2 mV/pH (Figure 26), close to the Nernst limit of 59.1 mV/pH at room temperature. The low noise of these devices allowed a change of 0.5 mV at the gate (Figure 34c) (which is equivalent to $8.4 \times 10^{-3}$ units of pH change) to be detectable with a signal to noise ratio of 2.60 ± 0.46 (averaged over 15 devices). A SNR of 1 would correspond to only $3.2 \times 10^{-3}$ units of pH change, very similar to the results obtained from the full noise analysis presented in Section 5.2.2.

7.2 PCR Detection

Previous studies have demonstrated the use of pH changes to monitor nucleic acid amplification using indicator dyes and bulk field effect transistors, for applications such as sequencing and specific sequence detection [3, 23, 117]. To demonstrate the feasibility of our nanoFET setup in measuring pH changes due to nucleic acid amplification, we performed PCR using a standard thermocycler and measured the pH using both standard phenol red pH indicator as a positive control and our nanoFET approach. The pH change generated from PCR amplification is a competing process between proton production and buffering. Typically, PCR amplification is performed in a highly buffered system, to prevent the proton production during amplification from changing the pH. However, we use a customized low buffered system to take advantage of the pH signal generated from
the process as a method of readout. We found that longer DNA templates (1074 base pairs) were used, as shorter sequences (<300 base pairs) did not generate a large enough pH change within each amplification cycle. This is because proton generation per PCR cycle is directly proportional to the length of the DNA strands produced. Using a model system to simulate what would happen in immuno-PCR, we pre-synthesized and purified a 1074 base pair biotinylated sequence from the pUC19 plasmid (see Appendix D for probe synthesis protocol) and performed PCR with non-biotinylated primers (see Appendix E for PCR protocol). The designed primer sequences are presented in Table 6. These primer sequences were selected out of 10 tested pairs.

Table 6. PCR primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Forward</td>
<td>CAA CAG CGG TAA GAT CCT TGA GAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAG TTC TTG AAG TGG TGG CCT AAC</td>
</tr>
</tbody>
</table>

For nanoFET measurements, approximately 15 µL of each samples was sequentially withdrawn from the PCR tubes into the microchannel over the nanoFETs at a flow rate of 20 µL/min, separated by small air gaps in between to prevent mixing of the solutions, and measured on the nanoFET setup. The change in surface potential was extracted as described in Equation 5, with $I_{DS}$ being the average current signal for each sample, and $I_{DS0}$ being the current signal for the no DNA sample after 30 cycles, and plotted in Figure 35a. Lower cycle numbers (30 cycles) do not amplify the smaller (<fM) initial DNA concentration samples enough to yield a measurable pH change, but at higher cycle numbers (55 cycles), the smaller initial concentration samples have excellent response (whereas the high concentration samples begin to saturate due to deactivation of
polymerase and depletion of consumables). At 55 cycles, a starting concentration of 30 aM is clearly detectable, and increasing the cycle number will further lower the detection limit, as PCR has been shown to have single copy sensitivity [118]. An example of the nanoFET real-time sensing data from a representative device for 30 cycles of amplification is shown in Figure 35c. The small air gaps between the samples cause temporary interruptions to the gating, occasionally introducing transient spikes, without affecting overall response to the sample. The pH steps show excellent repeatability in both directions, indicating that we do not have nonspecific adsorption of PCR components or cross-contamination between the samples as they are sequentially introduced through the microfluidic channel.

As a positive control to demonstrate that the signal we detect on our nanoFET sensors is indeed due to DNA amplification and its resulting pH changes, we performed fluorescence detection using ethidium bromide agarose gels (Figure 36) and colorimetric pH detection using phenol red indicator added to the amplification mixture. After PCR, the samples were injected into the Nanodrop 8000 Spectrophotometer to measure the absorbance of the pH indicator. Phenol red has absorbance peaks at around 433 nm and 560 nm, and the ratio of absorbance at 560 nm to 433 nm decreases as pH decreases. The ratios are shown in Figure 35b, with the y-axis inverted for ease of visualization, and a photograph of the samples are shown in Figure 35d. There is a good match between the nanoFET and the positive control optical data. Note that it is not surprising to see that thermocycling itself also has a significant effect in lowering the pH, which can be detrimental to the sensitivity and dynamic range. However this can be circumvented by
using an isothermal method of nucleic acid amplification, which would also result in a decrease in pH upon amplification [117].

Figure 35. nanoFET pH detection from PCR amplification and colorimetric verification. (a) nanoFET detection and (b) Nanodrop absorbance ratio at 30 and 55 cycles. Dotted line depicts signal level of the no DNA control. The error bars represent standard error of the mean (S.E.M.) from 3 Nanodrop measurements or 13 device measurements. (c) Representative example of a real-time trace from nanoFET detection at 30 cycles. (d) Representative photographs of samples after PCR amplification. Phenol red indicator allows for visualization of pH changes, and serves as positive control verification for nanoFET detection.
Figure 36. Gel verification and optical fluorescent detection of PCR products at 30 cycles. The band location matches the expected product size.

7.3 Immuno-PCR Detection

We now demonstrate this approach of using immuno-PCR to achieve ultrasensitive detection of proteins by nanoFET sensors in full serum. Here we use a serum biomarker, IL-2, as a model antigen. We chose IL-2 as it is an essential regulator in immunity homeostasis, autoimmunity, cancer, and infection [119], and because of the availability of robust commercial ELISA kits. The main challenge in immuno-PCR is the level of background introduced by non-specific adsorption during protein and DNA binding steps. Decreasing non-specific adsorption will drastically improve the detection limit of immuno-PCR. Several strategies have been devised to decrease the background, such as using pre-assembled detection antibody/DNA conjugates to increase sensitivity, reproducibility, and dynamic range [120]. Our goal here is to demonstrate the advantage
of an exponential amplification process for nanoFET detection of proteins over a linear method, rather than to optimize immuno-PCR itself. Therefore we chose the simplest method of stepwise coupling, in which the biotinylated detection antibody is attached to the biotinylated DNA strand using streptavidin. Our process is shown schematically in Figure 37a, and described in detail in Appendix B. Washes between each step help to lower background and ensure that PCR-interfering factors from the analyte sample are removed prior to amplification.

We measured the pH of the resulting products using the nanoFET sensors in the same manner as previously described. The surface potential change is extracted as described in Equation 5, with $I_{DS}$ being the average current signal for each sample, and $I_{DS0}$ the average current signal for the no IL-2 control. The no IL-2 control is prepared using the same procedures and at the same time as the rest of the samples, but using pure unspiked serum as sample. Therefore, any nonspecific adsorption, amplification, and pH changes due to thermocycling itself are encompassed in the control. 15 devices were measured and their average and standard errors of the mean are plotted in Figure 37b. To demonstrate the advantage of our exponentially amplifying detection system over the more commonly used linear system, we compared our immuno-PCR assay to an ELISA performed with urease readout (Figure 37b). Assuming a reliably discernable change of 0.5 mV in signal based on Figure 34c, a lower limit of $\sim$10-15 fM of IL-2 is detectable using our platform, and the signal is approximately linear up to $>200$ pM. Our nanoFET platform with immuno-PCR readout gives a detection limit that is at least $10^4$ fold better than nanoFET with ELISA-urease readout. The commercial IL-2 ELISA kit on which our
assay is based has a dynamic range between 133.3 fM – 13.3 pM (Figure 38 and shown in cyan in Figure 37), and the nanoFET detection using immuno-PCR has already surpassed this range in both the upper and lower limits by an order of magnitude, even without using pre-coupled detection antibody-DNA conjugates.

Figure 37. Assay comparison between exponential amplification (immuno-PCR) vs. linear amplification (ELISA-urease) for protein detection in serum with nanoFETs. (a) Procedure for stepwise coupling of DNA to antibody-antigen complex during immuno-PCR. (b) IL-2 detection through immuno-PCR. The error bars represent S.E.M. from 15 devices. (c) IL-2 detection through ELISA-urease (note different x axis scale). The error bars represent S.E.M. from 16 devices. The cyan region represents the dynamic range specified by the IL2 ELISA kit (see Figure 38).
Figure 38. Optical ELISA detection with commercial kit. The y-axis values are obtained by subtracting the absorbance at 570 nm from the absorbance at 450 nm. The error bars represent standard error of the mean from duplicate samples. The cyan region represents the dynamic range specified by the kit.

### 7.4 Conclusion and Future Outlook

We have demonstrated highly sensitive detection of IL-2 from serum from <20 fM to >200 pM by applying immuno-PCR to protein detection using nanoFETs. This indirect approach overcomes the issues commonly faced by nanowire detection to achieve protein sensing in full serum, and pushes its detection limit by employing an exponential amplification method. The approach can be readily applied to detect other analytes, for which the detection limit and specificity will depend on the affinities and cross-reactivity of the antibodies used. Further optimization of the immunoassay to reduce background using pre-conjugated components[120] or increase signal using additional amplifier approaches such as magnetic beads or nanoparticles [121, 122], can enhance the sensitivity of this assay. Due to real-time capabilities of nanoFETs, quantitative monitoring can be achieved by integrating heating elements into the sensing setup and allowing amplification to occur over the nanowires. Further improvements can
be made by using isothermal nucleic acid amplification methods instead of PCR to eliminate thermocycling. This system requires no optical components, and requires no dyes or additional factors that can potentially inhibit amplification. As the nanoFET readout is based on protons, which have a very high diffusivity, a stable signal is obtained almost instantaneously after the sample solution is introduced. Therefore, in future expansions, a large multiplexed assay can be read in a short amount of time. The demonstrated system is already portable, and can be further miniaturized (into a handheld form factor) with on-chip heater and sensors and integrated electronic design. Our approach for highly sensitive nanoFET-based detection of proteins allows the future development of universal and highly sensitive technology that can be applied to the detection of a wide variety of analytes, and enables point-of-care applications such as home care and field diagnostics.

* This work has been accepted to *Analytical Chemistry*. 
8.1 Background

The current version of pH-based immuno-PCR using nanoFETs requires many peripheral setups to complete. In addition to the nanoFET sensing setup, well strips and thermocycling equipment are also used to complete the assay. Many of these items have significant potential for miniaturization to enhance system portability. For example, the sensing board and data acquisition system can be mostly incorporated on-chip, as well as heating and temperature sensing components for thermocycling. In addition, the assay time and complexity can be significantly reduced by changing the nucleic acid amplification process to one that is isothermal. Immuno-PCR was used as a proof of concept, as PCR is well established and predictable; however, thermocycling is not only
algorithm intensive, but time-consuming due to the heating and cooling steps. Loop-mediated isothermal amplification (LAMP) is an isothermal amplification technique for DNA and RNA that rapidly amplifies the target sequence when the reaction is held at around 65-70 °C [123]. The LAMP process uses four to six primers that can specifically adhere to the template strand. One pair of primers, called inner primers, denoted FIP and BIP, are hybrid primers that bind to the F1-F2c and B1-B2c regions on the template strand respectively. Another pair of primers, called outer primers, denoted F3 and B3, bind to the F3c and B3c regions respectively. These four primers are shown in Figure 39a. Although effective amplification can be achieved with just these four primers (as shown in Figure 39b&c), an additional pair of primers, called loop primers, denoted loopF and loopB, can be designed to bind to the loop regions (e.g. between B1 and B2) on the 5’ to increase the number of starting points for amplification and thus the LAMP speed.

In order to improve our nanoFET immuno-PCR platform and move towards integrated, portable sensing, several main changes were made. Firstly, instead of doing a surface-based immunoassay in well-strips, a bead-based immunoassay is done, so that the antibody-antigen-DNA complex can be easily transferred into the detection well over the nanoFETS in the future to monitor amplification in real-time. This also allows the immunoassay to be done in a different vessel than amplification, reducing background due to nonspecific adsorption of reagents on vessel walls. Secondly, the PCR process was replaced with LAMP to take advantage of the aforementioned benefits of rapidity and simplicity. Thirdly, there is ongoing work to integrate the nanoFET sensing setup with a thin-film heater, but significant improvements are still needed, and is therefore not
included in this thesis. The ultimate goal is to incorporating the heating and temperature sensing on-chip with the nanoFETs.
Figure 39. Loop mediated isothermal amplification process. (a) Primers and starting structure with primer-binding regions. (b) Initial annealing and elongation steps that produce the first looped starting structure. (c) Subsequent cycling amplification steps to produce more elongated structures. Figure taken from [124].
8.2 LAMP Detection

The LAMP primers were designed using the PrimerExplorer software, to the same pUC19 DNA sequence used in our immuno-PCR experiments. We tested five sets of LAMP primer sequences, and selected one set of six primers due to high specific amplification speed and low nonspecific background. Their sequences are summarized in Table 7. The bead immuno-LAMP process is outlined in detail in Appendix C.

Table 7. LAMP primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tr>
<td>FIP</td>
<td>GGC GAG TTA CAT GAT CCC CCA TGA TCG GAG GAC CGA AGG A</td>
</tr>
<tr>
<td>BIP</td>
<td>TGA TCG TTG GGA ACC GGA GCT GTT GTT GCC ATT GCT ACA GG</td>
</tr>
<tr>
<td>F3</td>
<td>CGG CCA ACT TAC TTC TGA CA</td>
</tr>
<tr>
<td>B3</td>
<td>CGC CAG TTA ATA GTT TGC GC</td>
</tr>
<tr>
<td>LoopF</td>
<td>TTG TGC AAA AAA GCG GTT AGC</td>
</tr>
<tr>
<td>LoopB</td>
<td>AAT GAA GCC ATA CCA AAC GAC GAG C</td>
</tr>
</tbody>
</table>

As with PCR, in order to test the effectiveness of the amplification itself, different concentrations of starting DNA were spiked into the LAMP mastermix containing phenol red pH indicator. The LAMP mastermix recipe is detailed in Appendix F. Using the DNA Engine Tetrad 2 Thermocycler set at 70 °C, we carried out amplification of DNA only samples. The results are shown in Figure 40. One can see that the amplification is very efficient, requiring only tens of minutes compared to hours for PCR. Signal from the lowest non-zero concentration sample can be observed by the naked-eye at 30 minutes with very good contrast from the DNA-free control.
Figure 40. DNA-only LAMP amplification using optimized primer set.

8.3 Immuno-LAMP Detection

We performed the bead-immuno-LAMP process using the same ELISA kit used for immuno-PCR to detect IL-2 in serum, and flowed the resulting solutions over the nanoFET devices. The amplification was carried out for 17.5 minutes. The real-time data from a nanoFET obtained by flowing samples sequentially from low concentration to high concentration is shown in Figure 41a (due to sample limitation, as well as the presence of beads at the bottom of the tubes, we were not able to repeat the sample...
measurements from high to low), and the averaged data from all devices in a measurement is shown in Figure 41b. The photograph of samples containing phenol red indicator is also shown in Figure 41c for reference. One can see that the immuno-LAMP gave a very similar limit of detection as immuno-PCR with less than 20 minutes of amplification.

Figure 41. Protein detection in serum with nanoFETs. (a) Real time data from a single nanoFET device. (b) IL-2 detection through immuno-LAMP. The error bars represent S.E.M. from 16 devices. (c) Representative photographs of samples after immunoLAMP amplification. Phenol red indicator allows for visualization of pH changes, and serves as positive control verification for nanoFET detection.
8.4 Conclusion and Future Outlook

We have demonstrated highly sensitive detection of IL-2 from serum from ~30 fM to >30 pM by applying immuno-LAMP to protein detection using nanoFETs. The lower detection limit is comparable to immuno-PCR, but the dynamic range is decreased due to the faster amplification rate of LAMP. This should not pose an issue for detection, as the eventual goal of this method is to monitor the amplification in real-time using the nanoFETs, which will likely extend the dynamic range of this assay. Immuno-LAMP has all the aforementioned advantages of immuno-PCR, with the addition of being able to achieve rapid amplification in just minutes with no thermal cycling. Just like immuno-PCR, immuno-LAMP can be universally applied to the detection of any analyte, as long as a pair sandwich antibodies exist for that analyte. In the future, other isothermal amplification techniques can be tested, such as nucleic acid sequence-based amplification, multiple displacement amplification, rolling circle amplification, etc. [125], to determine the best method for this application. In addition, more work is needed to optimize the probe strand sequence, primer sequences, and mastermix composition to produce the most rapid amplification rate without compromising amplification specificity. Current work is on-going to integrate LAMP and immuno-LAMP with real-time nanoFET detection, by incorporating microheaters and temperature sensors into the nanoFET sensing setup. Microwells over the nanoFET devices will potentially allow multiple analyte concentrations to be simultaneously detected.
**SUMMARY AND OUTLOOK**

The primary focus of this thesis is on using nanoFETs to detect various interesting biological events through detecting pH. NanoFETs were chosen as the method of readout due to their low noise, low power, low cost, miniaturizability, and integratability. pH was chosen as a signal transduction mechanism due to the small size of protons and the lack of functionalization requirement for detection. Traditionally, macromolecular detection using field-effect-based devices suffers from Debye screening in high ionic strength solution, and other issues in general. However, the use of pH mitigates these effects while maintaining the advantages of having an electronic readout.

We first presented a noise spectroscopy analysis to motivate the selection of Al₂O₃ as our dielectric of choice for pH sensing. Comparing SiO₂ and Al₂O₃, we found that although SiO₂ offered a superior limit of detection in $\Delta \psi_0$ based on its noise, it had inferior signal, given by the pH sensitivity. On the other hand, although Al₂O₃ had a
worse noise level due to its poorer quality interface, it had a near Nernstian pH sensitivity. After taking into account the signal and the noise for both surfaces, we found that the minimum detectable $\Delta p$H for Al$_2$O$_3$ was about half of that of SiO$_2$, making Al$_2$O$_3$ the better choice for our application. It was interesting to note however that for applications which generate surface potential shifts that are relatively independent of dielectric material, SiO$_2$ may be a better choice. This conclusion only applies to the devices demonstrated in this thesis, and could easily change depending on the fabrication cleanliness, fabri

cator experience, and the fabrication flow in general. This further points to the importance of noise spectroscopy in device design and optimization on a case by case basis.

We then applied Al$_2$O$_3$-passivated nanoFETs to the demonstration of two areas of application: enzyme-substrate interaction quantification, and protein concentration detection. In the case of enzyme-substrate interactions, many reactions directly cause a change in the solution pH through the alteration of the substrate molecule, which can be sensed by an underlying nanoFET. We demonstrated the detection of substrate levels in PBS to below 200 $\mu$M (for enzyme-substrate interactions that net one OH$^-$ per substrate molecule consumed), the detection of enzyme activity in PBS and urine to 0.02 units/mL (for enzyme-substrate interactions that net one H$^+$ per substrate molecule consumed), and the monitoring of kinetics to extract the kinetic constant. This allows us to monitor certain classes of enzyme substrate interactions in real time, in a label-free manner, and can be easily calibrated between different solutions through their buffering capacities.

Although direct monitoring of reactions has the benefit of measuring activity in real-time, for our method, it is ultimately limited to the classes of reactions that can
directly produce a pH change. Frequently, one is simply interested in the concentration of a certain analyte in a sample. To achieve this, we presented an indirect method of protein detection, by converting its recognition to a pH response via immuno-PCR or immuno-LAMP. We achieved a detection limit on the order of tens of femtomolar for a model protein IL-2 in full serum in both immuno-PCR and immuno-LAMP, but with immuno-LAMP being much faster for amplification. We found that amplifying the sandwich immunoassay using an exponential process like nucleic acid amplification offered a much lower limit of detection compared to linear processes like enzyme catalysis. In both immuno-PCR and immuno-LAMP, we opted to detect proton production rather than the nucleic acid amplicons because of the faster kinetics of proton binding, which allowed us to read samples sequentially in a high throughput fashion, as well as the lack of functionalization requirements, which allowed chips to be reused.

Although we have presented proof-of-concept demonstrations of how biorecognition events can be detected directly and indirectly through measuring pH, much remains to be improved and optimized. Referring back to Box 1, there is a desire to engineer biosensors that has good specificity, a large dynamic, high sensitivity, excellent resolution and integratability. In Box 2, we briefly comment on each of these aspects for pH-based biomolecular sensing by FETs as an outlook to the future.
Box 2

**Specificity** – the specificity of a biosensor depends on the specificity of the biorecognition event, the specificity of analyte as an indication of a particular disease, the specificity of the amplification, and the degree of blocking. The first two are purely biological topics of research, and will not be commented on here. Specificity of amplification should be tested to ensure that adsorption of nonspecific sequences that may be present in the sample or blocking solution do not amplify and obscure the signal. In addition, the degree of blocking needs to be optimized for each application to ensure that nonspecific binding is prevented without reducing specific binding. For the pH readout itself, excessive blocking is typically not an issue as protons are small enough to reach the surface. However, any procedure involving reagent binding and signal generation in the same location (e.g. immuno-PCR) need to be carefully blocked to prevent as much nonspecific signal as possible.

**Dynamic range** – for most applications, there is a biochemically or clinically meaningful concentration range for the target analyte. For instance, for the cardiovascular disease marker cardiac troponin I, the range is from the normal serum level (<420 fM) to the peak level during myocardial infarction (2.1-4.2 nM) [126]. At minimum, a useful sensor would have a dynamic range that encompasses the target relevant range, but it is often preferred that the limit of detection is made as low as possible such that smaller volumes of samples can be taken and diluted. To achieve a low limit of detection, efforts need to be made both from biochemistry (e.g. increasing biorecognition affinity, reducing nonspecific adsorption, improving amplification efficiency) and device design (improving signal to noise ratio) aspects.

**Sensitivity** – higher sensitivity is often desired, as long as the strategies used to improve signal do not introduce additional noise. Biochemical improvements (e.g. increasing biorecognition affinity, improving amplification efficiency), and device design improvements (e.g. having larger binding area, more Nernstian surface) can both improve the sensitivity and the SNR of the platform. However, adding external amplification stages to improve sensitivity do not usually translate to a better performance, as the sensitivity improvements are often equal for both signal and noise, yielding the same SNR. Nevertheless, external amplification can be useful to ensure that the sensor output lies within the optimal range for the measurement electronics.

**Resolution** – the efforts to improve resolution should be very similar to those for the limit of detection, as the resolution is the limit of detection of change.

**Integratability** – the benefit of an FET setup mainly lies in the integratability. There is significant room for miniaturization of the current design, and other temperature control
and signal conditioning components can be designed on-board or on-chip to make an all-in-one platform that has the versatility to be adapted to many different applications. The platform can adopt a well-plate-like format in the future to measure multiple samples simultaneously.
Notes:

- This section provides the mask design of the various layers, as described in Section 4.1. Some features may not be visible in the zoomed out images.

- As each mask is shown, the relevant features for that layer are colored in pink, whereas all other layers are shown as outlines only.
Mask #1 – Alignment marks (dark field mask, features clear)

Figure 42. Locations of alignment marks on wafer (marked in red boxes)

Figure 43. 3.3 mm x 3.3 mm alignment mark die (left), and zoomed in view of an alignment mark (right).
Mask #2 – Backgate (dark field mask, features clear)

Figure 44. 6.6 mm x 6.6 mm die showing locations of backgate features. Each die contains eight backgate openings.
Mask #3 – Ion implantation (dark field mask, features clear)

Figure 45. 6.6 mm x 6.6 mm die showing locations of implantation (left), and zoomed in view of a nanoribbon device showing source and drain implantation (right).

Mask #4 – Nanoribbon definition (bright field mask, features opaque)

Figure 46. There are 32 nanoribbons per 6.6 mm x 6.6 mm die, located at the center (left), and zoomed in view of a nanoribbon device showing the mesa feature (right).

Mask #5 – Contact opening (dark field mask, features clear)
Figure 47. 6.6 mm x 6.6 mm die showing locations of contact opening to allow dielectric removal (left), and zoomed in view of a nanoribbon device showing source and drain dielectric removal in preparation for metallization (right).
Mask #6 – Al metallization (dark field mask, features clear)

Figure 48. 6.6 mm x 6.6 mm die showing aluminum metallization including contact pads (left), traces to the 32 devices at the center of each die (right top), and zoomed in view of a nanoribbon device showing aluminum metallization to the source and drain (right bottom).

Mask #7 – On-chip reference electrode metallization (dark field mask, features clear)

Figure 49. There are four on chip reference electrode lines per 6.6 mm x 6.6 mm die.
Figure 50. 6.6 mm x 6.6 mm die showing SU-8 vias to contact pads (left), on-chip reference electrodes (top right), and zoomed in view of a nanoribbon device showing window opening over the ribbon (right bottom).
**APPENDIX B: IMMUNO-PCR FOR NANOFET DETECTION PROTOCOL**

Notes:

- Washes are extremely important in this protocol as insufficient washing can result in high background due to amplification.

- It is important that when washing, the pipette tips do not touch the well walls.

1. Robostrips (Analytic Jena) for immuno-PCR are coated with 100 μL/well of 2 μg/mL IL-2 capture antibody (Affymetrix) in coating buffer overnight at 4 °C.
2. The wells are washed once for one minute with 200 µL/well of wash buffer (1x PBS, 0.05% Tween-20), and three times for one minute each with 1x PBS.

3. The wells are blocked with 200 µL of The Blocking Solution (Candor) at room temperature for 2 hours with high-speed shaking.

4. The wells are washed once for one minute with wash buffer.

5. Varying concentrations of IL-2 antigen (Affymetrix) are spiked into 100% FBS (Gibco), and 100 µL of each antigen solution is added to corresponding wells. The sample solution is incubated for 3.5 hours at room temperature with gentle shaking.

6. The wells are washed four times for one minute each with wash buffer.

7. 100 µL of 0.06 µg/mL detection antibody (Affymetrix) in diluent (1x PBS, 0.05% Tween-20, 3% biotin-free BSA) is added to each well and incubated for 1 hour at room temperature with gentle shaking.

8. The wells are washed four times for one minute each with wash buffer.

9. 100 µL of 1 µg/mL streptavidin (Thermo Fisher Scientific) in diluent is added to each well and incubated for 30 minutes at room temperature with gentle shaking.

10. The wells are washed six times for one minute each with wash buffer.

11. 100 µL of 40 nM biotinylated reporter DNA in diluent is added to each well and incubated for 30 minutes at room temperature with gentle shaking.
12. The wells are washed eight times for one minute each

13. 100 µL of PCR mastermix is added to each well on ice (same recipe as detailed in Appendix E, but without DNA), and 30 cycles of PCR is carried out.

14. After amplification, the sealed samples can be frozen at -80 °C until pH measurement, as CO₂ acidification of solution is negligible at such low temperatures. However, it is important that the sample be allowed to come back to room temperature in a sealed environment before measurement (for both phenol red absorbance measurements and nanoFET measurements), as the measured pH value is dependent on the sample temperature.
APPENDIX C: IMMUNO-LAMP FOR NANOFET DETECTION PROTOCOL

Notes:

- Washes are extremely important in this protocol as insufficient washing can result in high background due to amplification.

- For step 2, antibody bead coupling, an optimal coupling concentration is ~40 mg beads/mL. For coupling only ~5 mg of beads, this can be reduced to ~20 mg beads/mL. Our protocol uses only 10 mg beads/mL. We found this coupling concentration to give good results, and this allowed us to make freshly coupled beads before each immuno-LAMP experiment.
1. Dynabeads M280 (Thermo Fisher Scientific) are washed twice with 0.1 M borate buffer (pH 9.5). Washes are done by placing the tube containing beads on a magnet for 1 min, discarding the supernatant, removing the tube from the magnet and resuspending the beads in fresh buffer.

2. Resuspend the washed beads in concentrated capture antibody solution, using 20 µg antibody per mg beads. Supplement solution volume to a bead concentration of 1 mg beads/60 µL solution using 0.1 M borate buffer. Then, supplement solution volume such that the final bead concentration is 10 mg beads/mL using 3 M ammonium sulfate in 0.1 M borate buffer (pH 9.5).

3. Mix thoroughly, and allow the antibody to couple to the beads on an end-over-end rotator in 37 °C for 18 hours.

4. The beads are washed once with wash buffer (1x PBS, 0.05% Tween-20), and three times for with 1x PBS.

5. The beads are blocked with 1 mL of The Blocking Solution (Candor) at room temperature for 1 hour with end-over-end rotation.

6. The beads are washed twice with wash buffer (1x PBS, 0.05% Tween-20).

7. The beads are blocked with 1 mL of 3M ethanolamine at room temperature for 2 hours with end-over-end rotation.

8. The beads are washed three times with wash buffer, and then separated into different tubes, with each tube containing 100 µg of beads.
9. Varying concentrations of IL-2 antigen (Affymetrix) are spiked into 100% FBS (Gibco). The supernatant from each tube is removed and 1 mL of each antigen solution is added to corresponding tubes. The sample solution is incubated for 2 hours at room temperature with end-over-end rotation.

10. The beads in each tube are washed three times with wash buffer.

11. 100 µL of 0.06 µg/mL detection antibody (Affymetrix) in diluent (1x PBS, 0.05% Tween-20, 3% biotin-free BSA) is added to each tube and incubated for 1 hour at room temperature with gentle shaking.

12. The beads in each tube are washed three times with wash buffer.

13. 100 µL of 1 µg/mL streptavidin (Thermo Fisher Scientific) in diluent is added to each tube and incubated for 30 minutes at room temperature with gentle shaking.

14. The beads in each tube are washed four times with wash buffer.

15. 100 µL of 40 nM biotinylated reporter DNA in diluent is added to each tube and incubated for 30 minutes at room temperature with gentle shaking.

16. The beads in each tube are washed six times with wash buffer.

17. 50 µL of LAMP mastermix is added to each well on ice, and amplification is carried out by holding the tubes at 68 °C (See Appendix F for mastermix and LAMP recipes).
APPENDIX D: BIOTINYLATED REPORTER DNA SYNTHESIS AND PURIFICATION PROTOCOL

Notes:

- Biotinylated reporter DNA 1074 base pairs in length is synthesized from the pUC19 plasmid template using a biotinylated forward primer biotin-CAACAGCGGAAGATCCTTGAGAG, and a non-biotinylated reverse primer GAGTTCTTGAAGTGGTGGCCTAAC (Integrated DNA Technologies).

- Mastermix for polymerase chain reaction (PCR) amplification contains 1x Phusion polymerase buffer (New England Biolabs), 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 mM of each dNTPs (Takara), 0.5 ng/µL pUC19 plasmid
(Thermo Fisher Scientific), 0.02 units/µL of Phusion DNA polymerase (New England Biolabs), in ultrapure, DNase and RNase free Water (Sigma Aldrich). 64 reactions containing 100 µL each can be typically run in parallel in 200 µL PCR tubes to generate a large amount of amplified product.

- Prepare reactions on ice.

1. Amplification in a thermal cycler (DNA Engine Tetrad 2, BioRad) is carried out using the following conditions: 5 min of denaturation at 95 °C, followed by 40 cycles of 1 min at 95 °C, 1 min at 58 °C, 2 min at 72 °C. The reactions are held at 72 °C for 10 min at the end to complete the amplification process.

2. The amplified product from all 64 tubes is combined and purified using the QIAquick PCR purification kit (Qiagen). The procedure is as outlined in Qiagen’s Quick-Start Protocol. The purified DNA is eluted in DI water.

3. 1:3 volume ratio of loading dye is added to the eluted product and run under 150 V of applied voltage on a 0.8% agarose (Genemate/Bioexpress) gel containing 0.5 µg/mL ethidium bromide.

4. The band of desired product size (at 1074) is cut out under UV illumination, and extracted from the gel using the QIAquick gel extraction kit (Qiagen), as outlined in Qiagen’s Quick-Start Protocol.

5. The concentration of the obtained stock of purified biotinylated reporter DNA is measured using Nanodrop 8000 (Thermo Scientific), and the stock is stored at -20°C until use.
APPENDIX E: PCR AMPLIFICATION PROTOCOL

Notes:

- Each PCR sample consists of 100 µL of solution containing 0.4 µM of each primer, 0.4 mM of each dNTP (New England Biolabs), 1 mg/mL of ultrapure BSA (Thermo Fisher Scientific), 3 mM of MgCl₂ (New England Biolabs), 50 mM of KCl, 0.1% of Triton-X 100, 0.8 M of betaine (Sigma-Aldrich), 100 µM of phenol red (only in nanodrop and gel electrophoresis samples), 0.05 units/µL of Taq polymerase (Takara) and various amounts of starting target DNA concentration in DI ultrapure, DNase and RNase-free water (Sigma-Aldrich).

- All materials are purchased or dissolved in pure DI water.
Phenol red was added in some of the samples to provide a visual indication as well as a quantitative absorbance analysis of the pH change, but was left out of the samples for nanoFET measurement in case they interfered with sensing. Phenol red supplementation up to 0.3% (8.47 mM) was found to not interfere with amplification [127].

To tailor this protocol for immunoPCR, create the mastermix with DI water replacing DNA.

Prepare reactions on ice.

1. The ingredients (other than phenol red, Taq polymerase, and DNA if doing DNA-only experiments) are combined to form the mastermix, and the pH of the mix is tuned to approximately 8.5 using fine-increment pH paper (VWR) before adding in phenol red (only in some samples) and Taq polymerase.

2. The mastermix is divided into PCR tubes and various amounts of DNA are added to the individual tubes such that the final volume in each tube is 100 µL.

3. PCR is set to run using the following conditions: 10 min of denaturation at 95°C, 55 cycles of 30 s at 95 °C, 30s at 48 °C, and 2 min at 72°C.

4. Thermocycling is paused temporarily at the end of 30 cycles to remove the corresponding 30 cycle tubes, and the program is resumed for the 55 cycle tubes (if applicable).
APPENDIX F: LAMP
AMPLIFICATION PROTOCOL

Notes:

-The LAMP samples for DNA-only experiments consists of 25 µL of solution each, the LAMP samples for immunoLAMP consists of 50 µL of solution each.

-Each LAMP sample contains 1.6 µM each of FIP and BIP primers, 0.8 µM each of Loop-F and Loop-B primers, 0.2 µM each of F3 and B3 primers, 800 µM of each dNTP (Lucigen), 1mg/mL of ultrapure BSA (Thermo Fisher Scientific), 10 mM of MgSO₄ (Lucigen), 10 mM of KCl, 0.1% of Triton-X 100, 0.15 M of betaine (Sigma-Aldrich), 100 µM of phenol red, 1x of OmniAmp DNA
polymerase (Lucigen) and various amounts of starting target DNA concentration in DI ultrapure, DNase and RNase-free water (Sigma-Aldrich).

- All materials are purchased or dissolved in pure DI water.

- To tailor this protocol for immunoLAMP, create the mastermix with DI water replacing DNA.

- Prepare reactions on ice.

1. The ingredients (other than phenol red, OmniAmp polymerase, and DNA if doing DNA-only experiments) are combined to form the mastermix, and the pH of the mix is tuned to approximately 8.5 using fine-increment pH paper (VWR) before adding in phenol red and OmniAmp polymerase.

2. The mastermix is divided into PCR tubes (see notes at the beginning of this section) and various amounts of DNA are added to the individual tubes.

3. LAMP amplification is run with temperature held at 70 °C.

4. Stop the reaction after desired time has passed by placing the samples back on ice.
# Appendix G: Symbol and Acronym Definitions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Device area</td>
</tr>
<tr>
<td>$C_{ox}$</td>
<td>Dielectric capacitance per unit area</td>
</tr>
<tr>
<td>$C_{Si}$</td>
<td>Capacitance per unit area of silicon channel</td>
</tr>
<tr>
<td>$C_{dl}$</td>
<td>Capacitance per unit area of solution double layer at solution-dielectric interface</td>
</tr>
<tr>
<td>$C_{nanoFET}$</td>
<td>nanoFET capacitance per unit area</td>
</tr>
<tr>
<td>$f$</td>
<td>Frequency</td>
</tr>
<tr>
<td>$g_m$</td>
<td>Transconductance</td>
</tr>
<tr>
<td>$H^+_s$</td>
<td>Surface proton concentration in solution</td>
</tr>
<tr>
<td>$H^+_b$</td>
<td>Bulk proton concentration in solution</td>
</tr>
<tr>
<td>$I_{DS}$</td>
<td>Drain current</td>
</tr>
<tr>
<td>$I_{GS}$</td>
<td>Gate leakage current</td>
</tr>
<tr>
<td>$I_{DS0}$</td>
<td>Baseline current or current at a certain designated time = 0.</td>
</tr>
<tr>
<td>$I_c$</td>
<td>Ionic concentration</td>
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<tr>
<td>$k$</td>
<td>Boltzmann constant</td>
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<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$K_a, K_b$</td>
<td>Equilibrium constants</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
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<tr>
<td>$L$</td>
<td>Transistor channel length</td>
</tr>
<tr>
<td>$N_0$</td>
<td>Interface trap density</td>
</tr>
<tr>
<td>$N_s$</td>
<td>Total binding site density</td>
</tr>
<tr>
<td>$N_A$</td>
<td>Avogadro’s number</td>
</tr>
<tr>
<td>$Q_0$</td>
<td>Total charge at solution-dielectric interface</td>
</tr>
<tr>
<td>$\Delta Q_0$</td>
<td>Change in total charge at the solution-dielectric interface</td>
</tr>
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<td>$\Delta \text{pH}_{\text{min,comp}}$</td>
<td>pH resolution for comparison</td>
</tr>
<tr>
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<td>Elementary charge</td>
</tr>
<tr>
<td>$S_I$</td>
<td>Drain current noise power density</td>
</tr>
<tr>
<td>$S_{VFB}$</td>
<td>Flatband voltage noise power density</td>
</tr>
<tr>
<td>$S_{VG}$</td>
<td>Gate voltage noise power density</td>
</tr>
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<td>$[S]$</td>
<td>Substrate concentration</td>
</tr>
<tr>
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<td>Temperature</td>
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<tr>
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<td>Solution gate voltage</td>
</tr>
<tr>
<td>$V_T$</td>
<td>Threshold voltage</td>
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<td>Flatband voltage</td>
</tr>
<tr>
<td>$V_{DS}$</td>
<td>Drain voltage</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximum rate of product generation</td>
</tr>
<tr>
<td>$\Delta V_{\text{min}}$</td>
<td>Voltage resolution</td>
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<td>$\Delta Q_{\text{min,comp}}$</td>
<td>Charge resolution for comparison</td>
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<td>Transistor channel width</td>
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<tr>
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<td>pH sensitivity</td>
</tr>
<tr>
<td>$\delta I$</td>
<td>Root mean square current noise amplitude</td>
</tr>
<tr>
<td>$\varepsilon_0$</td>
<td>Vacuum permittivity</td>
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<tr>
<td>$\varepsilon_{dl}$</td>
<td>Relative permittivity of solution</td>
</tr>
<tr>
<td>$\varepsilon_{ox}$</td>
<td>Relative permittivity of dielectric</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Characteristic tunneling distance in dielectric</td>
</tr>
<tr>
<td>$\lambda_D$</td>
<td>Debye length in solution</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Carrier mobility</td>
</tr>
<tr>
<td>$\nu$</td>
<td>Initial rate of product generation</td>
</tr>
<tr>
<td>$\sigma_0$</td>
<td>Charge density at solution-dielectric interface</td>
</tr>
<tr>
<td>$\Delta \sigma_0$</td>
<td>Change in charge density at solution-dielectric interface</td>
</tr>
<tr>
<td>$\sigma_{ox}$</td>
<td>Charges in dielectric</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$\Phi_S$</td>
<td>Work function of semiconductor</td>
</tr>
<tr>
<td>$\Phi_M$</td>
<td>Work function of metal (or polysilicon) gate</td>
</tr>
<tr>
<td>$\Phi_{El}$</td>
<td>Work function of reference electrode</td>
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<tr>
<td>$\chi^{\text{sol}}$</td>
<td>Surface dipole moment of the solution</td>
</tr>
<tr>
<td>$\psi_{El}$</td>
<td>Potential drop at reference electrode-solution interface</td>
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<tr>
<td>$\psi_0$</td>
<td>Surface potential at solution-dielectric interface</td>
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<tr>
<td>$\Delta \psi_0$</td>
<td>Change in surface potential</td>
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<tr>
<td>$\psi_{dl}$</td>
<td>Surface potential drop across the solution double layer</td>
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<tr>
<td>$\psi_{\text{bulk}}$</td>
<td>Bulk potential of solution</td>
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</table>

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ALD</td>
<td>Atomic layer deposition</td>
</tr>
<tr>
<td>APTES</td>
<td>Aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>BOE</td>
<td>Buffered oxide etch</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNF</td>
<td>Cornell NanoScale Science and Technology Facility</td>
</tr>
<tr>
<td>DAQ</td>
<td>Data acquisition</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DIP</td>
<td>Dual in-line package</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FET</td>
<td>Field effect transistor</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
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<tr>
<td>ISFET</td>
<td>Ion-sensitive field effect transistor</td>
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<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
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<tr>
<td>LAMP</td>
<td>Loop mediated isothermal amplification</td>
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<tr>
<td>LCC</td>
<td>Leadless chip carrier</td>
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<tr>
<td>MOSFET</td>
<td>Metal-oxide-semiconductor field effect transistor</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>PCB</td>
<td>Printed circuit board</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PECVD</td>
<td>Plasma-enhanced chemical vapor deposition</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>RIE</td>
<td>Reactive ion etching</td>
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<tr>
<td>RMS</td>
<td>Root mean square</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
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<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
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<tr>
<td>SOI</td>
<td>Silicon-on-insulator</td>
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<tr>
<td>TAMRA</td>
<td>Tetramethylrhodamine</td>
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</tbody>
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REFERENCES


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