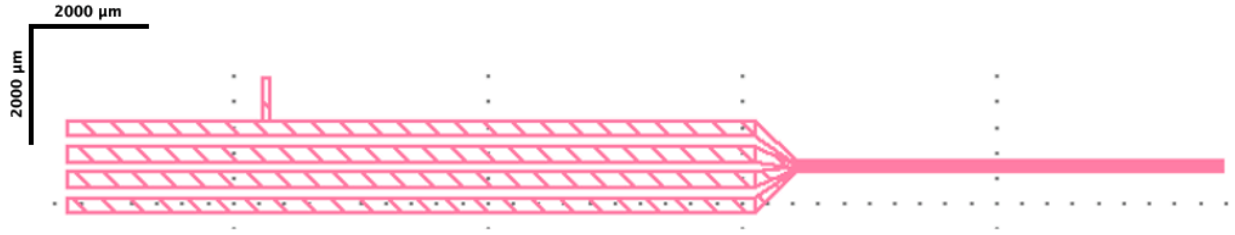
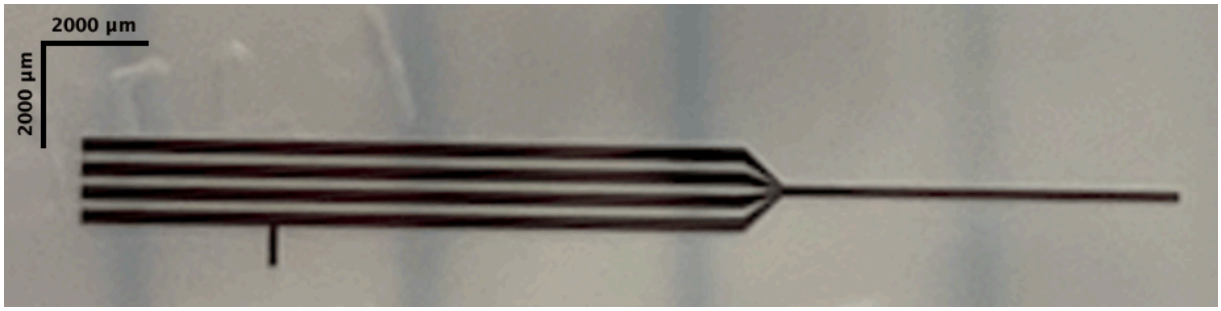
ENGR241 Final Report:

Microstructured Electrode Design for Enhanced Multiplex Electrochemical Aptamer Sensing Performance In-Vivo





Emma Kranich, Caoimhe Lyons
Spring 2025

Soh Lab Collaborators: Qitao Hu, Yihang Chen
Mentors: Swaroop Kommera, Hye Ryoung Lee, Tony Ricco

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1. Introduction

1.1 Background

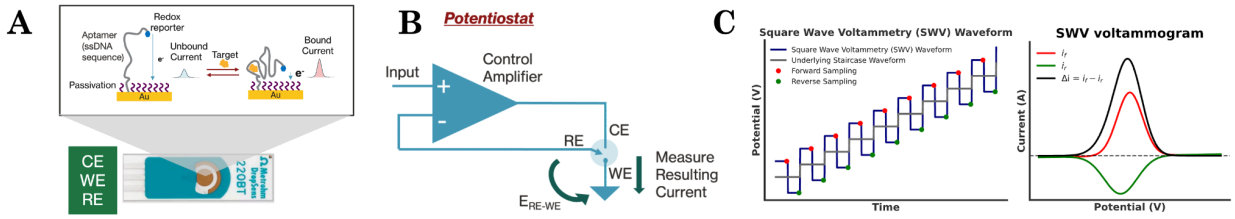


Figure 1: (A) Electrochemical aptamer switch, shown with a three electrode screen-printed electrode, (B) Block diagram of fundamental parts of a potentiostat, (C) Potential-time and current-potential plots associated with square wave voltammetry.

Continuous biosensors play a vital role in disease tracking and real-time health monitoring. Among these, electrochemical biosensors have shown promise for in vivo applications but have traditionally faced challenges with specificity and multiplexing. Aptamers, which are single-stranded DNA sequences that selectively bind to specific target molecules, offer an alternative to direct target redox readings. Upon binding to their target, aptamers undergo a conformational change, which can be transduced into an electrical signal when a redox-active reporter (e.g., methylene blue) is attached to the aptamer. This structural shift alters the proximity of the reporter to the electrode surface, modulating electron transfer and resulting in a measurable current change at the redox potential (see Figure 1A). The high specificity of aptamers enables electrochemical sensors to distinguish between closely related molecules, providing more accurate and reliable measurements than traditional sensing approaches that rely on direct target detection.

A standard electrochemical sensor setup involves a working electrode (WE) with the sensing element, a reference electrode (RE) that maintains a stable potential for comparison, and a counter electrode (CE) for the return current path. The system is controlled and measured using a potentiostat (see Figure 1B). The voltage potential is held between the reference and working electrodes, and the current is measured to read out the redox reporter proximity to the device surface (by detecting changes in electron transfer).

Square wave voltammetry (SWV), illustrated in Figure 1C, is a specific method of voltage stepping used to measure faradaic current while minimizing contributions from background non-faradaic processes. Square pulses of increasing potential are applied between the working and reference electrodes at a given frequency (or the frequency can be swept to find the optimal choice for maximum current change). The current is sampled at the end of each forward and reverse pulse after non-faradaic currents have decayed, and the two samples are subtracted to obtain the final differential current signal. This approach enhances sensitivity by suppressing non-faradaic effects and emphasizing faradaic current, which directly reflects redox reporter activity. This makes SWV well-suited to aptamer-based systems, where subtle conformational shifts drive signal changes. By contrast, traditional techniques like cyclic voltammetry (CV) sweep voltage continuously and often record more significant background current, making it less sensitive for detecting low-abundance analytes or subtle shifts in redox behavior.

1.2 Project Goals

To develop an optimal electrochemical aptamer-based continuous sensor, our design focused on tackling four key issues in sensor implementation:

1. **Achieving ultrasensitive detection:** Many physiologically relevant biomarkers appear at nanomolar or sub-nanomolar concentrations in biofluids. Detecting these low concentrations requires maximizing signal generation from a limited number of target binding events. To address this, we designed nanoporous electrodes with high surface area for dense aptamer functionalization[1].
2. **Resisting biofouling:** Biofouling presents a significant challenge for in vivo sensors, as prolonged contact with biological fluids leads to nonspecific adsorption and signal degradation. In addition to offering increased surface area, nanoporous architectures provide physical shielding for surface-bound aptamers, reducing their exposure to fouling agents. This protective feature makes nanoporous designs particularly well-suited for long-term applications[2].
3. **Enabling multiplexing:** Many clinical decisions require monitoring multiple biomarkers simultaneously to build a more comprehensive picture of physiological state. To enable this, we integrated several working electrodes and independent signal pathways into a compact layout. This approach enables multiplexed sensing without the spectral limitations of traditional fluorescent probes.
4. **Miniaturizing the device for tissue implantation:** Lastly, to minimize tissue damage and reduce measurement artifacts, we prioritized miniaturization of the sensor geometry. Smaller electrodes are less invasive and better suited for accurate, long-term implantation in biological environments.

1.3 Benefit to SNF

This project demonstrates an application of Stanford Nanofabrication Facility (SNF) capabilities for the development of miniaturized electrochemical aptamer sensor probes. Through our work, we aim to contribute to the broader SNF community in several ways:

1. **Guidelines for fabricating functional electrochemical probes for in vivo use:** The fabrication process, which includes bilayer resist lithography, metal deposition, and nanoporous gold (np-Au) patterning, is tailored for small-form-factor probe structures. The design considerations can inform future biosensor designs at SNF. These workflows can serve as a guide for other users seeking to fabricate implantable or electrochemical sensors using SNF infrastructure.
2. **Instructions for aptamer functionalization and electrochemical interrogation:** The project includes protocols for immobilizing aptamers on gold surfaces and performing square wave voltammetry (SWV) measurements. These protocols offer a foundation for users unfamiliar with biosensor surface chemistry and electrochemical readout methods to begin exploring aptamer-based sensing approaches.
3. **Enabling interdisciplinary applications:** This work highlights the potential of SNF to support projects at the interface of engineering, chemistry, and biology. By showing how microfabrication tools can be adapted for biologically relevant applications, this project helps broaden the perception of SNF as a resource for biosensing research. It encourages new users from medical device development to view SNF as accessible and adaptable to their research needs.

2. Process Development and Experimentation

2.1 Design Choices

Probe Layout

The probe layout (KLayout shown in Figure 2) was developed to support standard three-electrode electrochemical measurements, requiring a working electrode (WE), counter electrode (CE), and reference electrode (RE). To enable multiplexed sensing, a second working electrode was incorporated into the design, allowing simultaneous detection of multiple analytes.

The electrode shafts taper toward the sensing region, which is intended for precise in vivo implantation. At the opposite end, the wider pads are sized to align with a 4-pin flat flexible cable (FFC) connector, providing compatibility with standard interfacing hardware.

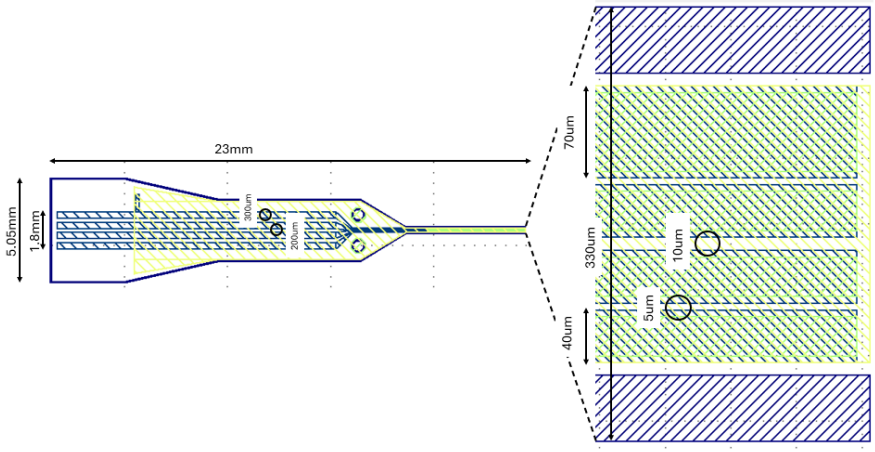


Figure 2: KLayout diagram of full probe design including dimensions. This KLayout file includes multiple layers for nanoporous probe and planar connections as well as passivation layers. Proof of concept probe fabricated was only one layer of four electrodes (blue, shown as pink in cover image).

All four electrodes are fabricated using nanoporous gold (np-Au), as described in the subsequent fabrication section (Section 2.2). To realize an on-probe reference electrode (RE), gold in the RE region is electroplated with silver, which is subsequently chlorinated to form Ag/AgCl (see Section 2.2.B). This enables stable, integrated reference potential generation without the need for an external reference electrode.

Substrate Choice

Silicon was initially considered as a substrate for this device. However, due to the frequency of signals used in SWV for aptamer interrogation (potentially exceeding 1 kHz) silicon was ultimately avoided. This decision was made to minimize parasitic capacitance that can arise between metal traces, the silicon dioxide (SiO_2) insulating layer, and the conductive silicon substrate, which could distort electrochemical measurements.

Instead, Pyrex 7740 borosilicate glass was selected as the substrate. This material eliminates the parasitic capacitance concern by removing the conductive substrate layer altogether. While Pyrex lacks the well-defined crystal structure of silicon, making it more difficult to scribe and cleave cleanly, its electrical insulation properties are more suitable for high-fidelity SWV-based sensing, making it a better fit for this application.

Patterning Choice

A bilayer lift-off process was selected to define the metal electrodes, using LOL2000 as a lift-off underlayer and SPR220-3 as the photoresist. SPR220-3 was selected due to its ability to form thicker resist layers than alternatives like SPR3612, ensuring that the resist height remains greater than the deposited metal thickness. This is critical for avoiding edge bridging and enabling clean lift-off.

The bilayer structure creates a slight undercut in the resist profile, which facilitates lift-off by allowing solvent access to the underlying sacrificial LOL layer. This helps prevent unwanted metal retention and ensures the final electrode features remain well-defined.

Further details of the fabrication process are provided in Section 2.2.

2.2 Fabrication Process Flow

2.2.A Full Fabrication Flow

The flow in Figure 3 shows the process for fabricating and functionalizing a multiplexed electrochemical aptamer sensor probe.

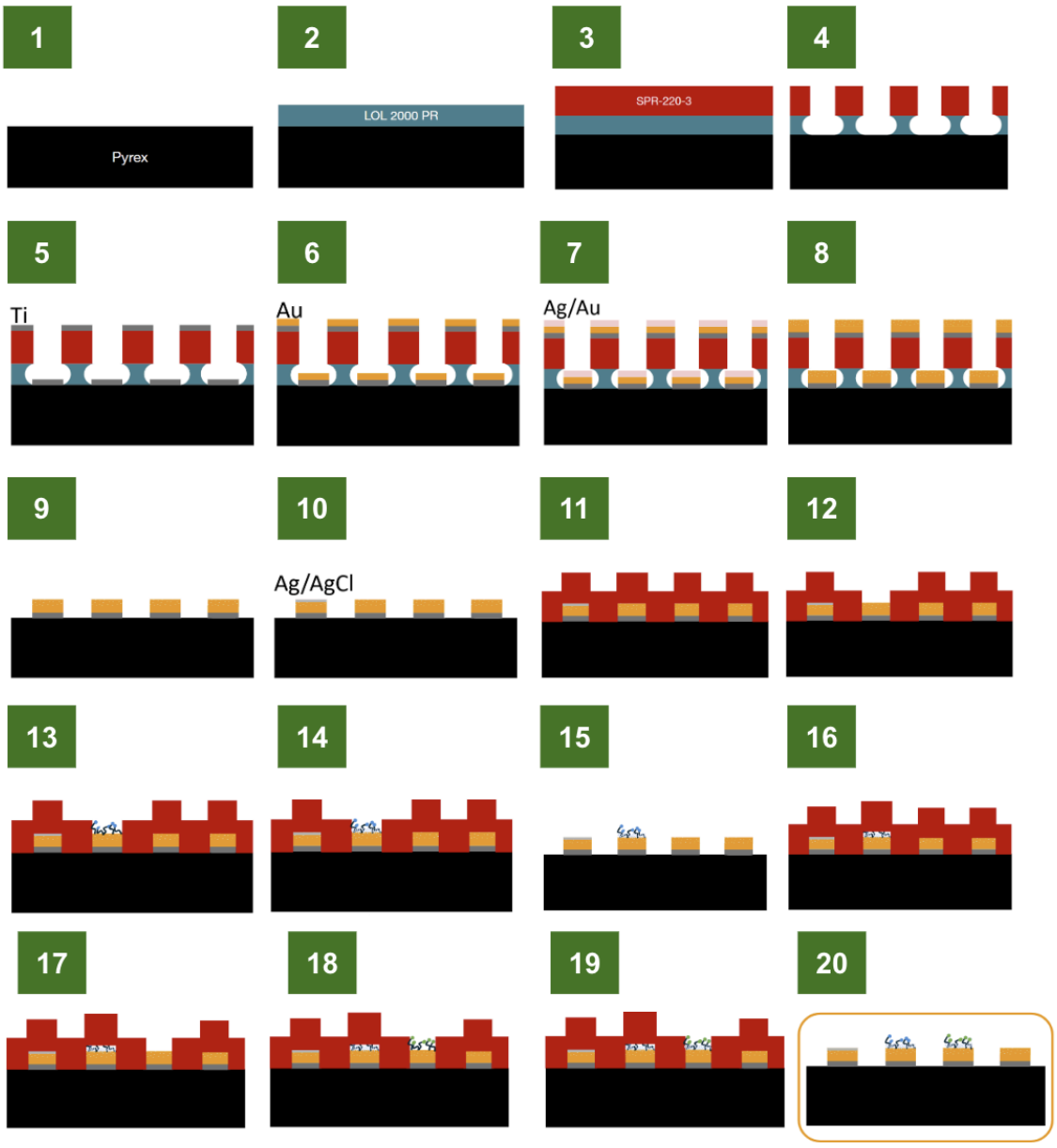


Figure 3: Full fabrication flow for nanoporous gold, multiplexed electrochemical aptamer-functionalized probe.

The process steps (greater detail on individual steps in Section 2.2.B):

1. Clean Pyrex 7740 water (acetone, isopropanol, dry with nitrogen).
2. Spincoat and soft bake LOL2000.
3. Spincoat and soft bake SPR220-3.
4. Expose electrode pattern, run post-exposure bake and develop in MF-26A.
5. Sputter deposit Ti.
6. Sputter deposit Au.
7. Co-sputter deposit Ag/Au.
8. Etch using 70% nitric acid to form nanoporous gold.
9. Liftoff resist using Remover 1165 and acetone.
10. Electroplate one of the electrodes to form an Ag/AgCl reference electrode.
11. Spin coat with resist and soft bake.
12. Expose one working electrode (WE1) in the design, run post-exposure bake and develop.
13. Functionalize the exposed working electrode (WE1) with aptamer.
14. Attach mercepto-hexanol passivation layer to the exposed working electrode (WE1).
15. Lift off resist with acetone.
16. Spin coat with resist and soft bake.
17. Expose the second working electrode (WE2) in the design, run post-exposure bake and develop.
18. Functionalize the exposed working electrode (WE2) with aptamer.
19. Attach mercepto-hexanol passivation layer to the exposed working electrode (WE2).
20. Lift off resist with acetone.

2.2.B Process Flow for One Working Electrode

Figure 4: Fabrication flow for patterning one np-Au electrode.

Wafer Preparation

1. Clean Pyrex 7740 wafer with acetone and isopropanol, and dry with nitrogen.
2. Run YES oven recipe 1 (HMDS vapor prime).
   Note: We saw resist adhesion issues when using only 150°C singe of YES oven recipe 5. The four electrode traces maintained definition with recipe 1 (Figure 5A), but traces were lost when recipe 5 was used (Figure 5B).

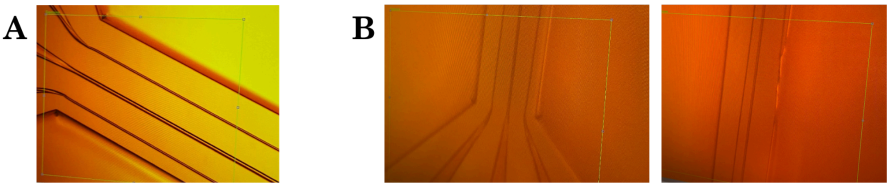


Figure 5: Comparison of feature definition post-development for (A) YES oven recipe 1, and (B) YES oven recipe 5.

Lithography

1. Spin Coat LOL2000 on Headway3 (2000 rpm for 45 s).
2. Soft bake 170°C for 4 min.
3. Spin coat SPR220-3 on Headway3 (2000 rpm for 45 s).
4. Soft bake 115°C for 60 s.
5. Exposure on Heidelberg2 (dose = 300 mJ/cm^2, defocus = -2).
6. Develop in MF26-A (120 s), rinse in deionised water.

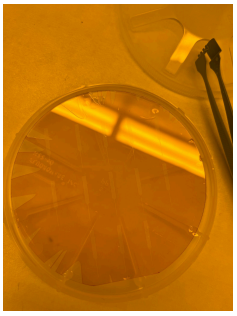


Figure 6: Wafer after lithography steps completed.

Deposition

1. Sputter 10 nm of Ti using Lesker Sputter.
2. Sputter 90 nm of Au Lesker Sputter.
3. Co-sputter 140 nm/80 nm of Ag/Au using Lesker Sputter.

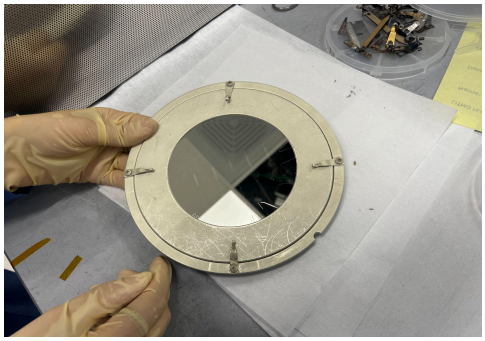


Figure 7: Wafer after deposition steps completed.

Etch and Liftoff

1. Etch with 70% Nitric Acid (7 min) to form nanoporous gold from co-sputtered gold/silver alloy.
2. Liftoff using Remover 1165 and acetone.

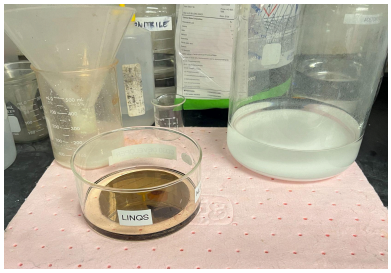


Figure 8: Wafer in nitric acid for np-Au etch process.

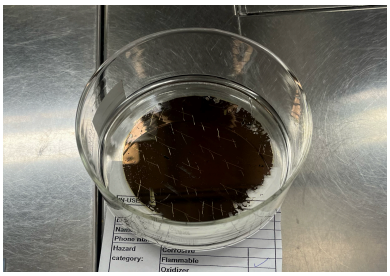


Figure 9: Wafer in acetone during liftoff.

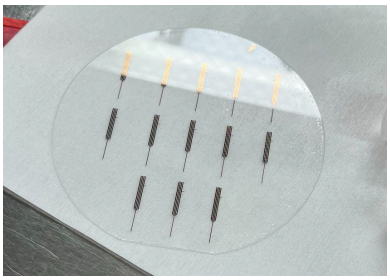


Figure 10: Wafer in acetone during liftoff.

Electroplating on Reference Electrode

To fabricate an on-probe Ag/AgCl reference electrode, a two-step process is employed: first, silver is electroplated onto the reference electrode site; second, the silver is chlorinated to form silver chloride.

The electroplating process uses a solution of 0.05 M potassium dicyanoargentate(I), based on established protocols such as those from Metalor Technologies[3] and Alan Blair[4].

Chronoamperometry is performed using a three-electrode setup, with the RE pad on the probe as the working electrode (WE). Silver is deposited via the following half-reaction:

$$Ag\,(CN)_2^- + e^- \rightarrow Ag + 2CN^-$$

After sufficient silver is deposited, the probe is immersed in household bleach (sodium hypochlorite solution) to convert the metallic silver into silver chloride via:

$$Ag + NaClO \rightarrow AgCl + Na^+$$

Wafer Breaking

To separate individual devices from the Pyrex wafer, a manual scribing and breaking method was used. The wafer was scored along a desired break line using a diamond scribe. It was then balanced across a pair of tweezers, aligned with the scribe mark, and gentle downward pressure was applied on either side to initiate the fracture.

Achieving clean, consistent breaks in Pyrex proved challenging due to the material's brittleness and lack of crystalline structure. Long, straight breaks and precise definition of small device geometries were difficult to maintain. However, by incorporating adequate spacing between devices in the layout, it was feasible to isolate individual probe regions with reasonable success.

Figure 11: Example break of Pyrex wafer

Electrical Connection Points

Electrical connection to the larger pads was done by hand-soldering wires onto the end. Alternatively, an FFC connector can be used (our design sized the large pads for optional connection to this type).

Aptamer Functionalization

1. Clean electrodes using cyclic voltammetry (CV) in H_2SO_4
    - Electrodes are cleaned using CV in 100 µL of H_2SO_4. The CV method is run until the voltammogram reaches a consistent threshold (our indicator was the lower peak reaching -0.4 V). The acid is removed and the electrode is dried with nitrogen. This cleaning step removes surface contaminants by reducing/oxidizing them off the electrode.
    - **Note:** This cleaning procedure was applied to commercial 220-BT screen-printed electrodes used for aptamer testing and was not required for fabricated electrodes on the custom probe.
2. Aptamer preparation
    - To reduce disulfide bonds and expose the thiol group for gold binding, tris(2-chloroethyl) phosphate (TCEP) is added to the aptamer at a 1000:1 molar ratio (TCEP:aptamer). The solution incubates in the dark at room temperature for 1 hour.
    - The reduced aptamers are then diluted to a final concentration of 1 µM in 1xPBS + 2 M NaCl.
3. Aptamer immobilization
    - Apply 50 µL of the 1 µL aptamer solution to each electrode. Electrodes are incubated at 4°C in a wetbox for 5 hours to allow aptamer immobilization onto the gold surface via thiol-gold bonding.
    - After incubation, electrodes are rinsed with 1xPBS and dried with nitrogen.
4. Surface passivation with MCH
    - To passivate unbound gold regions and minimize non-specific interactions, 50 µL of 5 mM 6-mercapto-1-hexanol (MCH) is applied to each electrode and incubated overnight in a fume hood. MCH backfills the surface, forming a dense self-assembled monolayer (SAM).
    - Following MCH incubation, electrodes are rinsed with 1xPBS and dried with nitrogen. 50 µL of 1xPBS is left on each electrode until use.

2.3 Square Wave Voltammetry Aptamer Testing

Using SWV, the performance of aptamers for kanamycin and dopamine was investigated. Kanamycin is an antibiotic used to treat bacterial infections and tuberculosis, typically administered at therapeutic doses in the 100s of micromolar range. Dopamine is a neurotransmitter that plays a key role in the brain's reward system and exists in vivo in the nanomolar to micromolar range. While for later demonstration, we functionalized the custom probe, the aptamer tests were run using commercial 220-BT Metrohm screen-printed electrodes.

Square Wave Voltammetry Protocol:

1. Choose voltage range around the chosen redox reporter peak. In this case, methylene blue was used, so the voltage sweep was from -0.5 V to 0.0 V.
2. Run a frequency sweep to find the largest percentage change in current (as in Figure 12).
3. Run square wave voltammetry at the frequency from step 2 with various concentrations of the target.

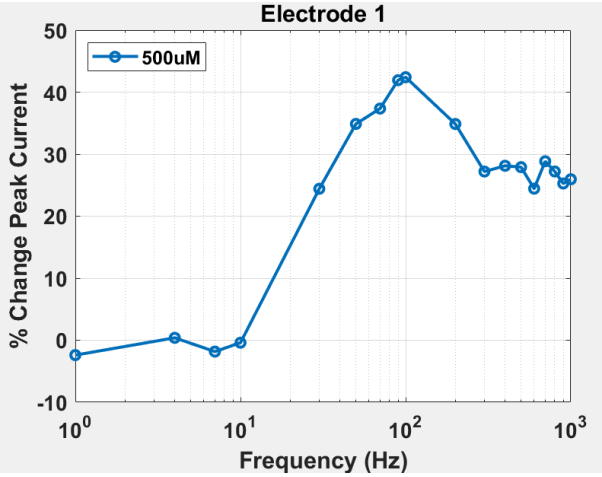


Figure 12: Peak current change vs. frequency for dopamine aptamer (baseline vs 500 µM dopamine target).

Square wave voltammetry parameters:

$t_{equilibration}$	E_{begin}	E_{end}	E_{step}	**Amplitude**	**Frequency**
1 s	-0.5 V	0.0 V	0.001 V	0.025 V	From Step 2 above

Aptamer Testing Results

For the kanamycin aptamer, 400 Hz was identified as an effective interrogation frequency, yielding a strong signal change with increasing analyte concentration (Figure 13A). The aptamer also showed clear reversibility, suitable for continuous sensing, upon repeated buffer washing and target addition (Figure 13B).

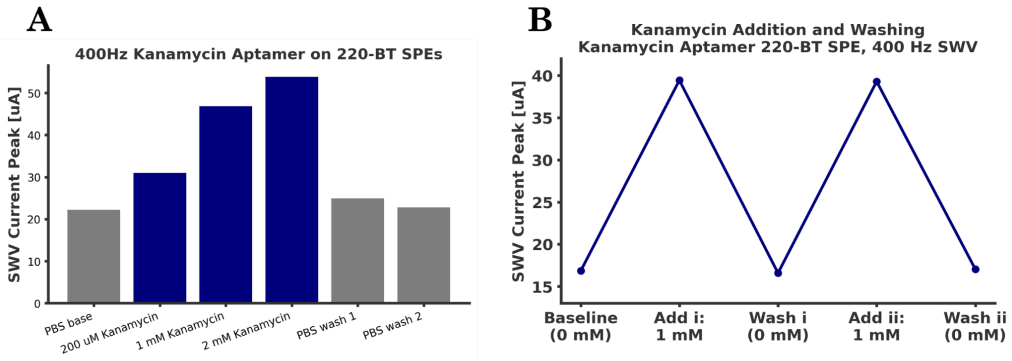


Figure 13: Kanamycin aptamer performance (400 Hz). (A) SWV current peak for buffer, target, and wash conditions, (B) SWV current peak for repeated target addition and washing.

For the dopamine aptamer, SWV measurements at 200 Hz and 100 Hz demonstrated target sensitivity and reversibility (Figures 14A and 14B, respectively). Both frequencies showed evidence of signal recovery after washing.

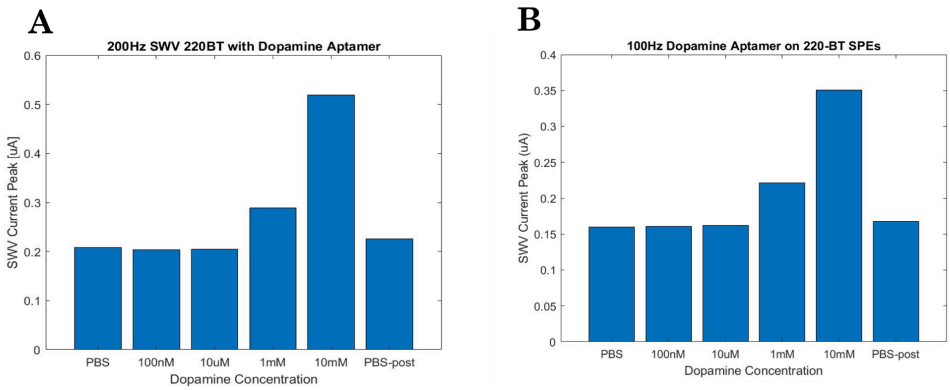


Figure 14: Dopamine aptamer performance. (A) 200 Hz SWV current peak for buffer, target, and wash conditions, (B) 100 Hz SWV current peak for buffer, target, and wash conditions.

These results confirm that the kanamycin and dopamine aptamers currently available in the lab are appropriate for functional testing of the fabricated devices.

2.4 Multiplexing Method

To evaluate whether photoresist processing interferes with aptamer function and redox signaling, aptamer-functionalized electrodes - previously characterized using (SWV) - were covered with photoresist (SPR220-3), then subjected to a removal process using acetone. The goal was to test compatibility with future multiplexed electrode patterning steps where individual sensors may need to be masked.

The full multiplex process is:

1. Spincoat SPR220-3 on Headway3 (2000 rpm for 45 s)
2. Soft bake 115°C for 60 s
3. Exposure on Heidelberg2 (dose = 300 mJ/cm2,defocus = -2) to open working electrode to functionalize next.
4. Develop in MF26-A. Developer will not disturb the aptamer-functionalized electrode because it will still be coated in photoresist.
5. Follow functionalization process (see steps 2-4 of Aptamer Functionalization in Section 2.2.B).
6. Remove photoresist with approximately 6s of acetone exposure (until photoresist is visually removed).

In our test, we used 220BT screen-printed electrodes, functionalized with dopamine aptamer. A control electrode (E1), not coated with photoresist and not exposed to acetone, was carried alongside the treated electrodes (E2, E3) through the same handling, and lighting conditions (e.g., nanofab, aptamer testing lab).

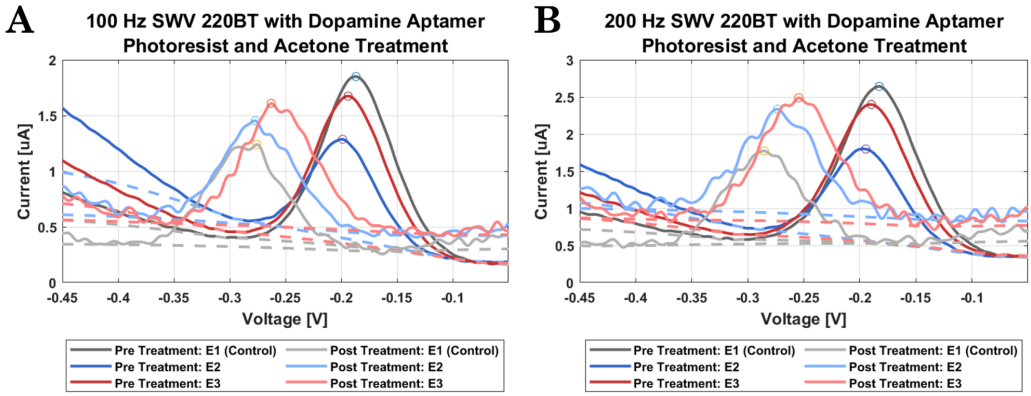


Figure 15: Photoresist dopamine aptamer multiplexing test. (A) SWV at 100 Hz, (B) SWV at 200 Hz.

After removal of the photoresist with acetone, SWV was repeated on all electrodes. From the results in Figure 15, a noticeable shift in peak potential was observed on both treated and control electrodes. We hypothesize that this may be due to light exposure affecting the methylene blue redox label, but additional testing is required to isolate the cause.

Despite the peak shift, the persistence of a clear redox signal after treatment indicates that the aptamer remained immobilized and functionally intact. This suggests the aptamer is robust to photoresist and acetone exposure under the conditions tested.

For future optimization, we propose:

- Testing alternative photoresists and spin conditions (e.g., thickness variation).
- Varying acetone exposure time.
- Including specific dark controls to evaluate the role of photo-induced degradation.

3. Probe Testing

The final test was conducted using a fabricated nanoporous gold probe functionalized with a kanamycin aptamer, following the procedure outlined in Section 2.2. The probe was connected to a PalmSens potentiostat and measured using square wave voltammetry (SWV) at 400 Hz.

As shown in Figure 16A, the device exhibits clear concentration-dependent signal changes and reversible behavior across repeated measurements, demonstrating successful fabrication, aptamer immobilization, and electrochemical functionality.

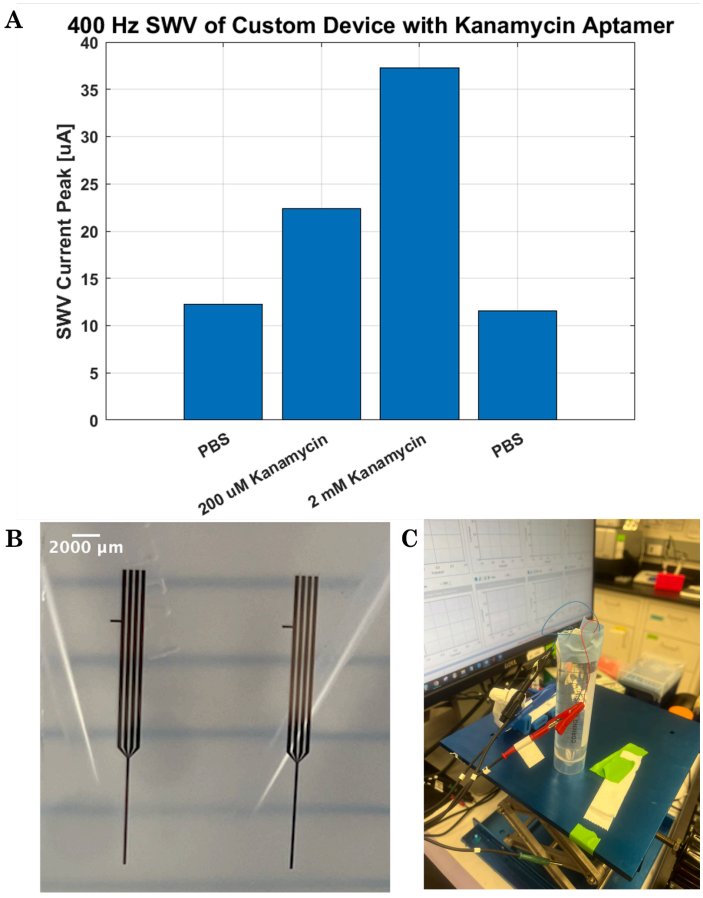


Figure 16: Final probe test (A) 400 Hz measurement results, (B) Fabricated final probes, © Measurement set up with connection to potentiostat and probe in solution.

4. Conclusion and Future Work

This project successfully demonstrated the design, fabrication, and functional testing of a miniaturized electrochemical aptamer-based probe using nanoporous gold electrodes. The sensor showed clear target-dependent signal changes, good reversibility, and compatibility with SWV-based interrogation-validating its potential for in vivo biosensing applications.

Future testing will include characterization of different photoresists for multiplexing as well as photoresist thicknesses and acetone removal time.

Additionally, fabrication on Pyrex was chosen for its insulating properties, but was difficult to cut out adequately small device sizing. As a future direction, we hope to fabricate with polyimide as the substrate for small device size. We will need to optimize adhesion and lithography steps for this substrate change. For insertion, we plan to test using PEG to connect the polyimide probe with a silicon probe. PEG will then be dissolved and removed after insertion, leaving the probe.

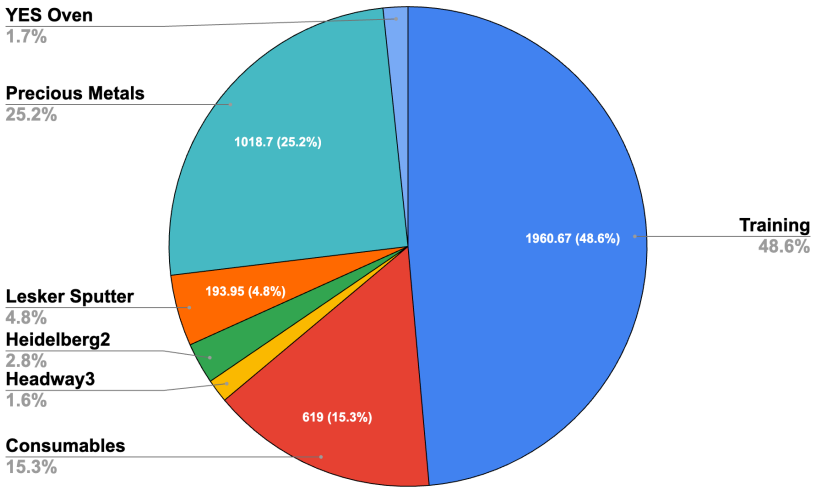
Finally, further work needs to be completed on the aptamer development itself. We need to test cross-reactivity and replace the aptamers with two diagnostically useful targets.

5. Acknowledgements

Thank you to our class mentors: Swaroop Kommera, Hye Ryoung Lee, and Tony
We also thank the Stanford Nanofabrication Facility (SNF) staff for their technical support, and our ENGR241 classmates for helpful discussions and camaraderie throughout the quarter.
This work was supported by Stanford University and the Stanford Nanofabrication Facility through the ENGR241 course.

6. Budget

Item	Amount $
Training	1960.67
Consumables	619
Headway3	63.41
Heidelberg2	111.86
Lesker Sputter	193.95
Precious Metals	1018.7
YES Oven	67.56
Total	**4035.15**



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