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Nano-confinement based platforms for screening bio-recognition elements and multiplexed sensing

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Tools for assessment of biomarkers of human performance and augmentation to enhance resilience to stress for promoting vigilance among field personnel of the Department of Defense is a central vision of AFOSR. This requires platforms capable of identifying receptors capable of recognizing key neurochemical human performance biomarkers and platforms to monitor biomarker expression profiles under perturbations. Using nanoscale confinement strategies to alleviate diffusional transport limitations, this project seeks to develop biofunctionalized material and device platforms for selecting receptors based on chemical binding affinity characteristics to biomarkers and signal amplification schemes to enhance detection sensitivity. This joint US-Taiwan project funded by AFOSR (US) and MOST (Taiwan) involved the Swami group at University of Virginia focused on detection platforms and the Chou group at Academia Sinica, Taiwan, focused on receptor screening platforms, while AFRL's 711th Human Performance Wing (Chavez group) supported the team on biomarker selection. Key collaborative highlights include microfluidic integration of nanoporous gold electrodes for redox amplification (Electrochimica Acta 2019, 318, 828-836) and its application towards sensitive detection of secreted metabolites for rapid determination of minimum bactericidal concentration levels (Sens. Act. B: Chem. 2020, 312, 127936); nanoconfinement to enhance crystal growth for creation of high aspect ratio defect-free porous metal organic framework crystals that enable selective enrichment of analytes (Nanoscale Advances 2019, 1, 2946-2952), nano-confined fluidics for screening receptors to biomarkers (Sens Act B: Chem 2018, 274, 338-342; Macromolecules 2020, 53, 1001-1013), including for SARS COV2 spike proteins (Scientific Reports 2021, 11 (1), 1-10); novel optofluidic (Biosens. Bioelectron. 2019, 142, 111545) and acoustic impedance detection methods (ACS Sensors 2021, 6, 3765-3772) and impedance-based cytometry and isolation of bacteria (Biosens. Bioelectron. 2020, 166, 112440) and apoptotic bodies (Adv. Biol. 2021, 2100438) and extracellular vesicles (Anal Chem 2019, 91 (16), 10424-10431). Key patent application include: the multiplexed control of impedance cytometry (PCT/US2019/053242) and real-time control of acoustic resonance frequency variations in microfluidic devices (U.S. Provisional Application 63/261,133). Follow-up work is focused on alleviating fluid transport to nanoconfined structures by developing on-demand nano-confinement approaches and applying these approaches to assess bacterial constructs for advancing synthetic biology.

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Final Report for FA2386-18-1-4100: Nano-confined platforms for screening and detection of human performance biomarkers – Nathan S. Swami (US PI), Chia-Fu Chou (Taiwan PI)

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ABSTRACT

Tools for assessment of biomarkers of human performance and augmentation to enhance resilience to stress for promoting vigilance among field personnel of the Department of Defense is a central vision of AFOSR. This requires platforms capable of identifying receptors capable of recognizing key neurochemical human performance biomarkers and platforms to monitor biomarker expression profiles under perturbations. Using nanoscale confinement strategies to alleviate diffusional transport limitations, this project seeks to develop biofunctionalized material and device platforms for selecting receptors based on chemical binding affinity characteristics to biomarkers and signal amplification schemes to enhance detection sensitivity. This joint US-Taiwan project funded by AFOSR (US) and MOST (Taiwan) involved the Swami group at University of Virginia focused on detection platforms and the Chou group at Academia Sinica, Taiwan, focused on receptor screening platforms, while AFRL's 711th Human Performance Wing (Chavez group) supported the team on biomarker selection. Key collaborative highlights include microfluidic integration of nanoporous gold electrodes for redox amplification (*Electrochimica Acta* **2019**, 318, 828-836) and its application towards sensitive detection of secreted metabolites for rapid determination of minimum bactericidal concentration levels (*Sens. Act. B: Chem.* **2020**, 312, 127936); nanoconfinement to enhance crystal growth for creation of high aspect ratio defect-free porous metal organic framework crystals that enable selective enrichment of analytes (*Nanoscale Advances* **2019**, 1, 2946-2952), nano-confined fluidics for screening receptors to biomarkers (*Sens Act B: Chem* **2018**, 274, 338-342; *Macromolecules* **2020**, 53, 1001-1013), including for SARS COV2 spike proteins (*Scientific Reports* **2021**, 11 (1), 1-10); novel optofluidic (*Biosens. Bioelectron.* **2019**, 142, 111545) and acoustic impedance detection methods (*ACS Sensors* **2021**, 6, 3765-3772) and impedance-based cytometry and isolation of bacteria (*Biosens. Bioelectron.* **2020**, 166, 112440) and apoptotic bodies (*Adv. Biol.* **2021**, 2100438) and extracellular vesicles (*Anal Chem* **2019**, 91 (16), 10424-10431). Key patent application include: the multiplexed control of impedance cytometry (PCT/US2019/053242) and real-time control of acoustic resonance frequency variations in microfluidic devices (U.S. Provisional Application 63/261,133). Follow-up work is focused on alleviating fluid transport to nanoconfined structures by developing on-demand nano-confinement approaches and applying these approaches to assess bacterial constructs for advancing synthetic biology.

1. RESEARCH OBJECTIVES AND STRUCTURE

Architectures of porous nanomaterials and slit-shaped fluidic devices that are designed for nanoscale confinement in one or more dimensions exhibit near instantaneous diffusion along the confined directions, while also advancing detection schemes based on plasmonic interactions with adsorbed materials and signal amplification from redox events. These phenomena have far-reaching implications in fields ranging from crystallization of porous nanomaterials to biomimetic recognition to communication of physical devices with engineered biosystems. The current project seeks to develop nanoconfined material and device approaches for screening receptors to human performance biomarkers and enable their detection per the following objectives and organization (**Fig. 1**):

(1) Receptor screening: nanoconfined porous material and fluidic strategies for creating bio-recognition elements to biomarkers based on binding affinity, with no diffusional limitations.

(2) High-sensitivity detection schemes: develop nanoconfined biomaterials for enabling redox amplification, nanostructured gratings for surface plasmon resonance detection, and impedance-based sensing secreted bodies.

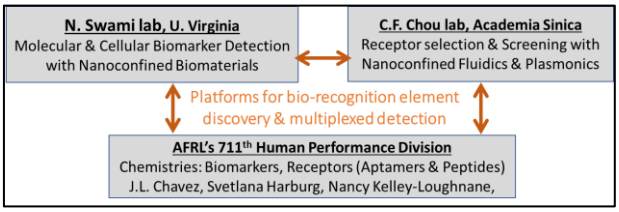

Fig. 1: Task Organization for this project

2. RESEARCH OUTCOMES

2.1. Nanoconfined fluidic platform for receptor screening (Lead – Chou group, Academia Sinica): Problem Statement: Current methods for selection of receptors use SELEX (systematic evolution of ligands by exponential enrichment), wherein the library of aptamer receptors is incubated with the target molecule, so that unbound aptamers can be washed away versus those bound with greater affinity to the target, for subsequent elution from the target molecule and PCR amplification.

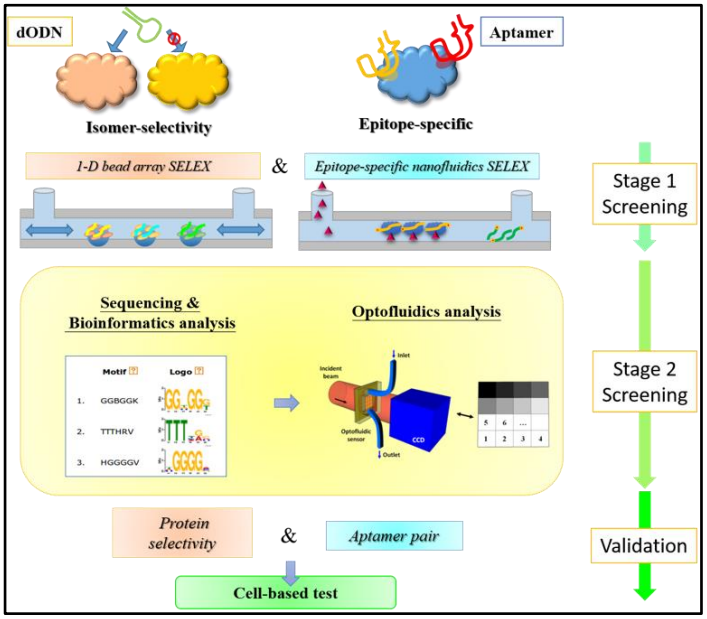

Fig. 2. Overview schematics for receptor sreening

After several rounds this process can eventually obtain highly enriched aptamers that have high affinities to the target of interest. However, key issues remain as stumbling blocks in the application of these synthetic receptors, including: (1) Lack of rapid screening methods to compare the binding affinities of aptamer candidates to the target protein, (2) High false-positive rates in diagnostics due to interferences, and (3) Need to improve sensitivity of aptamer-based platforms for multiplexed *in vitro* diagnostics. Hence, we seek to develop platforms to accelerate selection of the appropriate aptamer by utilizing the fundamental differences in chemical binding constants between various aptamer candidates to the target of choice, with diffusional limitations playing no significant role on the net binding kinetics. Specifically, using nanoscale confinement, we seek to characterize how fast the aptamer ligand binds (association rate, k_{on}), how fast it dissociates (dissociation rate, k_{off}), how specific is the ligand-target interaction (specificity), and how strong is the ligand-target binding (dissociation constant, K_d). Per Fig. 2, our vision is that these advances will enable epitope-specific nanofluidics SELEX and 1-D bead array SELEX strategies for aptamer identification. Preliminary results are shown in Fig. 3 and Fig. 4 for determining binding parameters for a selected aptamer to thrombin.

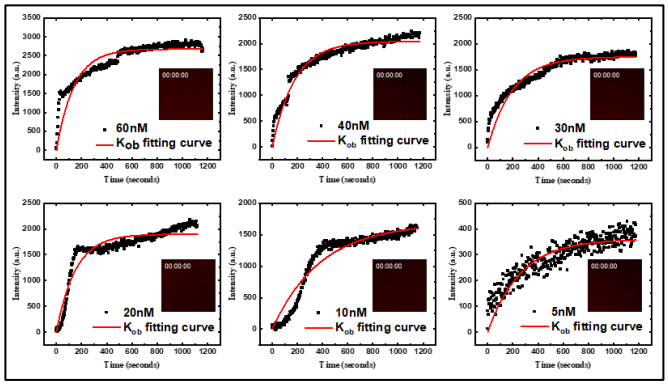

Fig. 3: Nanofluidic fluorescence microscopy for one-shot determination of binding kinetics of thrombin aptamer.

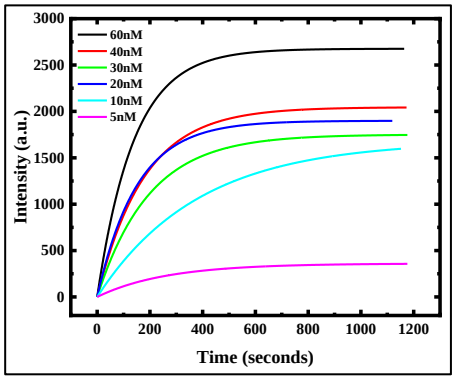

Fig. 4: A wide range of receptor concentration levels are used to determine binding kinetics

Based on this nanoslit confinement approach, we determined kinetic parameters in a one-shot assay with no need for repetitive wash steps and compare the numbers in Table 1 versus more laborious techniques. In follow-up work, we seek to transition from the nanofluidic fluorescence microscopy used herein to a method based on surface plasmon resonance-based imaging (SPRI) for label-free determination of binding.

2.2. SPRI detection system for high throughput aptamer screening (Lead – Chou group at Academia Sinica in collaboration with P-K Wei): Based on a device configuration per **Fig. 5** that includes nano-gratings for surface plasmon resonance imaging over a 1 mm by 1 mm array, the aptamer binding kinetics can be monitored in a high throughput manner, per example in Fig. 6. We envision that this platform would advance selection processes that allow to systematically *converge the random DNA sequence library to a limited number of leads, with* SPR image system providing an efficient way to characterize the binding properties of the leads for optimizing affinity and specificity.

Table 1 – Comparison of determined binding constants

Sequence	buffer	Method	k_{on}	k_{off}	K_D	referance
5'-**GGT TGG TGT GGT TGG**-CAA CC-3'	10 mM Tris–HCl, 140mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, pH 7.4	home-built SPR	5.2E+05	8.9E-02	1.7E-07	Colloids and Surfaces B: Biointerfaces, 88 (2011) 552
5'-**GGT TGG TGT GGT TGG**-CAA CC-3'	20 mM Tris–HCl, 140mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2,pH 7.4	QCM	3.3E+09	1.3E+02	3.9E-08	Bioelectrochemistry, 70 (2007) 127
5'-**GGT TGG TGT GGT TGG**-CAA CC-3'	10 mM Tris-HCl, pH 7.5	FRET (molecular beacon)	--	--	1.0E-08	Analytical Biochemistry,294 (2001)126
5'-**GGT TGG TGT GGT TGG**-TTT ATC AGC GTT CGA TGC TTC CGA CTA ATC AGC CAT ATC AGC TTA CGA CTA-3'	10 mM Tris-HCl,140 mM NaCl, 0.05% Tween 20, 50 µM EDTA, 50 µM EGTA, pH 7.4	FRET (switchSENSE)	3.3E+07	4.3E-02	1.3E-09	Molecules, 24 (2019) 2877
5'-thiol-TTT TTT TTT TTT TTT TTT TTT TTT T-**GGT TGG TGT GGT TGG**-3'	1XPBS(Gibco™ PBS)/0.05% Tween 20, pH 7.4	Nanoslit	6.4E+04	3.4E-03	5.3E-08	This work

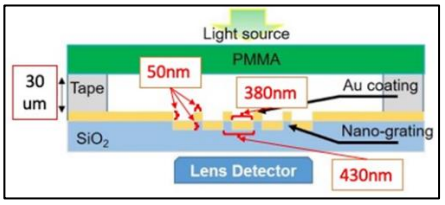

Fig. 5: Device configuration for surface plasmon resonance imaging (SPRI) with nano-grating to quantify binding kinetics

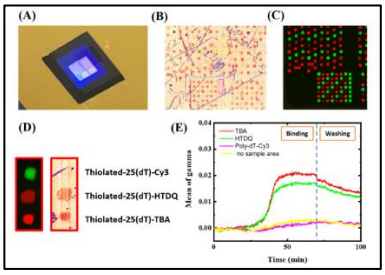

Fig. 6: High-throughput & label-free SPRI for profiling aptamer-thrombin interaction. (A) SPRI chip. (B) SPR image of microarray with aptamers (blue scratches are packaging defects). (C) Fluorescence image of the microarray. (D) SPRI (Right) and fluorescence images (Left). (E) Kinetic plots from (D).

2.3. Porous metal organic framework nanomaterials as receptors: (Lead – N. Swami, U. Virginia) Another receptor platform that we explored is based on nanoporous metal organic frameworks (MOFs), synthesized in the localized nanoconfined channel in presence of the target molecules of interest. In this manner, defect-free crystals imprinted with the target molecules of interest can be utilized as receptors for selectively enriching small molecule targets. Given the high porosity of the MOFs, they can enable localized 3D interactions of the target with the sensor. Per **Fig. 7**, *HKUST-1* MOFs synthesized in the nanochannel were loaded by diffusion of small molecules from the micro/nanochannel interface to allow for the inclusion of anthracene and methylene blue, whereas rhodamine is excluded. To enable chemical selectivity, the MOF crystallization was conducted in the presence of melatonin to imprint the pores and then washed (by solvents: dimethylsulfoxide → ethanol → acetonitrile). Upon diffusional loading with fresh of melatonin, its adsorption is apparent from attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) (**Fig. 8**). The transmittance peaks (normalized to the highest peak) shows that the secondary amide peak from melatonin (highlighted) is much greater for the HKUST-1 imprinted with melatonin than the unmodified MOF.

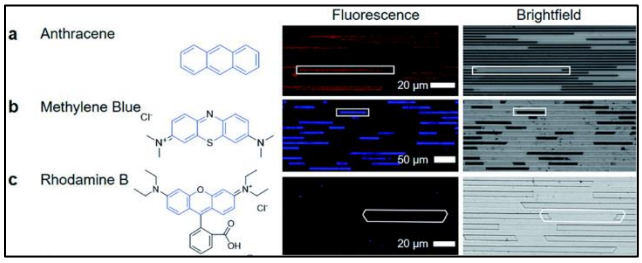

Figure 7: The HKUST-1 MOF enables inclusion of: a) Anthracene (co-location of fluorescence and the transmission brightfield signal) b) Methylene (blue fluorescence overlaps brightfield images to show that methylene blue is present in the crystal in a quantity to make the crystal appear darker in the brightfield image). c) Exclusion of Rhodamine B from the MOF.

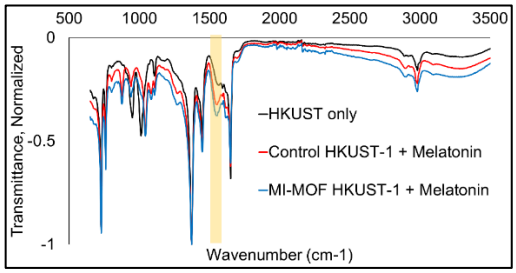

Figure 8: MOF molecularly imprinted to adsorb melatonin in HKUST-1 shows higher loading of melatonin compared to the control HKUST-1. The highlighted peak at 1554 cm^{-1} represents the secondary amide peak in melatonin.

2.4. Microfluidic integration of nanoconfined redox capacitors for biosensing signal amplification (Lead – N. Swami, U. Virginia): Redox processes utilize specific molecular species to shuttle electrons to and from the biological entity and the electrode, thereby mediating transduction between the target, its receptor, and the electrode. In this project, we developed biomaterial-based redox capacitors (**Fig. 9A**) that can amplify the redox-cycling current through interaction of the mediator pair with the redox capacitor film, for mediators with redox potentials bracketing that of the capacitor (**Fig. 9B**). Furthermore, by integrating the redox capacitor inside the architecture of nanoporous gold (NPG), the spatial extent across the capacitor film depth that is available to the redox mediator for electron transfer interactions with the electrode before escape into the bulk film is enhanced, thereby enhancing the redox amplification due to the significantly higher capacity of the nanoporous architecture versus on conventional substrates (**Fig. 9D vs. 9C**). Also, through microfluidic integration of nanoporous gold array (**Fig. 10A**), we can deliver continuous cues to the redox capacitor, while retaining its high detection sensitivity due to lowered impedance (**Fig. 10B**).

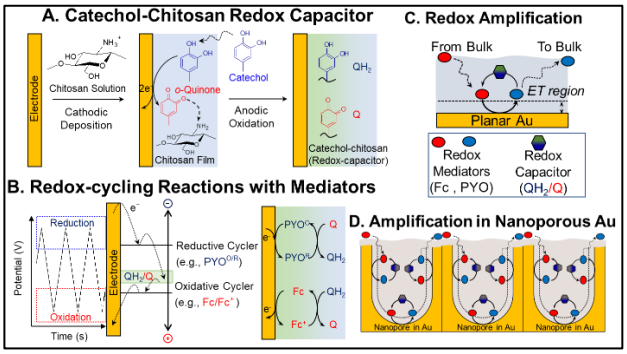

Fig. 9: A – Redox Capacitor; B – Redox Amplification; comparing planar (C) vs. nanoporous gold (D)

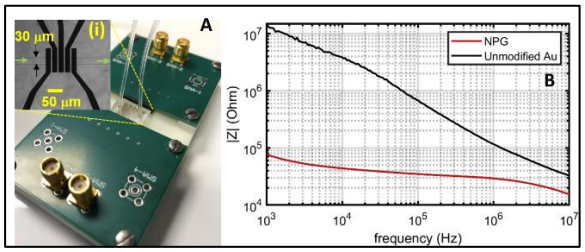

Fig. 10: A - Microfluidic integration of nanoporous gold electrode array for delivering continuous cues to the redox capacitor for sensing (see inset (i)). B – Enhanced sensitivity due to high capacitance and low impedance of nanoporous vs. planar gold

Possible applications of this strategy for multiplexed assessment of engineered bacterial constructs designed for human performance biomarkers (**Fig. 11**) and to screen peptide receptors designed for spike proteins, including SARS-Cov2 (**Fig. 12**), are presented. Such biofabricated nanoconfined platforms can be applied for specific and amplified detection of biomarkers, in the absence of diffusional limitations.

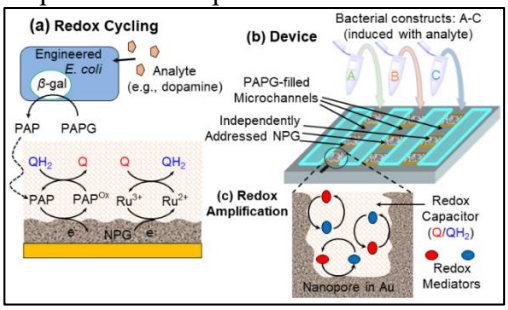

Figure 11: Biofabricated redox capacitor on nanoporous gold (NPG) for amplified detection in a microfluidic platform

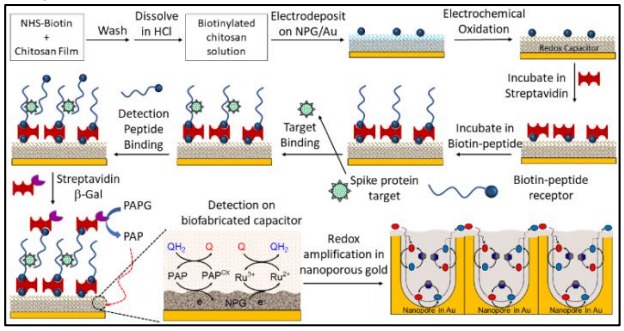

Figure 12: Biofabricated redox capacitor on nanoporous gold for amplified detection of SARS COV2 spike protein

2.5. Biophysical cytometry platforms for label-free monitoring of single-cell phenotypes (Lead – N. Swami, U. Virginia): Cellular biophysical properties offer a unique window to determine cell identity, health and disease pathogenesis. The emerging area of biophysical cytometry, wherein label-free single cell analysis is conducted based on electrical, mechanical and imaging modalities, can complement the biochemical information on cell protein expression profiles obtained from flow cytometry after fluorescent staining. This can advance studies on cellular phenotypic heterogeneity, wherein small variations in the proportion of subpopulations can fundamentally determine disease presentation, progression and treatment response. However, biophysical cytometry typically yields data of low dimensionality, which limits its application. Multiparametric approaches for high throughput biophysical analysis of single cells coupled to

machine learning models for automated phenotypic classification of its information rich content can unleash the potential of biophysical cytometry. In this project, in order to quantify the phenotypes of brain organoids, bacteria and apoptotic bodies, we developed multifrequency impedance-based flow cytometry coupled with dielectric models to obtain a biophysical picture of each subcellular region and with machine learning to train convolution algorithms for phenotypic classification (Fig. 13A-13D).

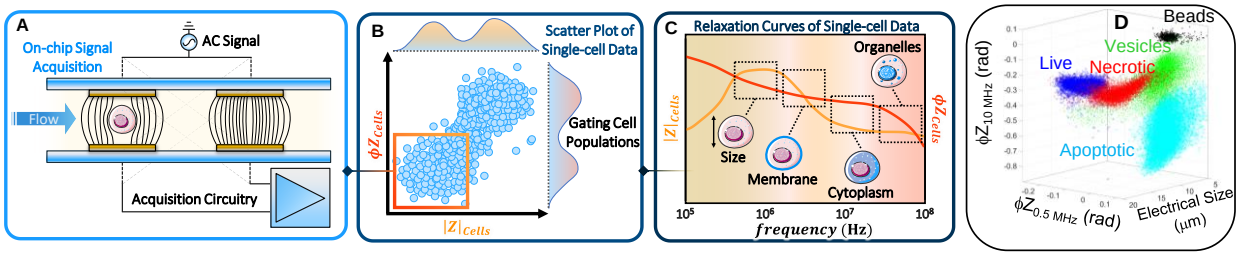

Fig. 13: (A) Impedance cytometry; (B) Data gating; (C) Multifrequency biophysical analysis; (D) Cell death pathway classification

3. RESEARCH TRAINING AND DISSEMINATION

3.1. Supported Personnel: This award was used to support graduate student: Ms. Armita Salahi, postdoc: Yi Liu and the PI: Nathan Swami.

3.2. Collaborative Publications: Following is a listing of the publications from this contract, many of which were based on collaborations between the Swami group (Univ of Virginia), Chou group (Academia Sinica) and AFRL's 711th Human Performance Wing (Chavez group).

1. "Multiplexed assessment of engineered bacterial constructs for intracellular β-galactosidase expression by redox amplification on catechol-chitosan modified nanoporous gold", Yi Liu, John Moore, Svetlana Harbaugh, Jorge Chavez, Chia-Fu Chou, Nathan S. Swami*, *Microchimica Acta* (accepted, in press)

2. "A High-Throughput Pipeline for Design and Selection of Peptides Targeting the SARS-Cov-2 Spike Protein", Monica Wolfe, Sean Webb, Yaroslav Chushak, Rachel Krabacher, Yi Liu, Nathan S. Swami, Svetlana Harbaugh, and Jorge Chavez, *Scientific Reports* (2021), 11 (1) 1-10, DOI: 10.1038/s41598-021-01225-2 <www.nature.com/articles/s41598-021-01225-2> (Impact Factor =5.1; Times Cited = 0.

3. "Real-time detection and control of microchannel resonance frequency in acoustic trapping systems by monitoring amplifier supply currents", Vahid Farmehini, Sadie Kiendzior, James P. Landers, Nathan S. Swami*, *ACS Sensors* (2021) 6 (10), 3765-3772. https://pubs.acs.org/doi/full/10.1021/acssensors.1c01580 ; Impact Factor =7.33; Times Cited = 0.

4. "Apoptotic bodies in the pancreatic tumor cell culture media enable label-free drug sensitivity assessment by impedance cytometry", Carlos Honrado, Sara Adair, John H. Moore, Armita Salahi, Todd Bauer, Nathan S. Swami, *Advanced Biology* (2021) 2100438. https://doi.org/10.1002/adbi.202100438 . Impact Factor =4.05; Times Cited = 0.

5. "Minimum bactericidal concentration of ciprofloxacin to Pseudomonas aeruginosa determined rapidly based on pyocyanin secretion", Y. Liu+, J. H. Moore, G. L. Kolling, J. S. McGrath+, J. A. Papin, N. S. Swami*; *Sensors and Actuators B: Chemical* (2020), 312, 127936. Impact Factor =7.1; Times Cited = 6

6. "Quantifying bacterial spore germination by single-cell impedance cytometry for assessment of host microbiota susceptibility to Clostridioides difficile infection", J. H. Moore, A. Salahi, C. Honrado+, C. Waburton†, C. A. Warren, Swami, N.S.*, *Biosensors and Bioelectronics* (2020), 166, 112440. Impact Factor =10.25; Times Cited = 10.

7. "Nanoconfinement-Induced DNA Reptating Motion and Analogy to Fluctuating Interfaces", JW Yeh, A Taloni, KK Sriram, JP Shen, DY Kao, CF Chou, *Macromolecules* (2020) 53 (3), 1001-1013.

8. “Spectral contrast imaging method for mapping transmission surface plasmon images in metallic nanostructures”, MY Pan, DK Yang, CY Lai, JH Weng, KL Lee, LC Chen, CF Chou, PK Wei. *Biosensors and Bioelectronics* (2019) 142, 111545

9. “Crystallization of High Aspect Ratio HKUST-1 Thin Films in Nanoconfined Channels for Selective Molecule Uptake”, S. Guthrie, L. Huelsenbeck, A. Salahi, W. Varhue, N. Smith, X. Yu, L. U. Yoon, J. J. Choi, N. S. Swami, G. Giri, *Nanoscale Adv.* (2019), 1, 2946-2952. Journal Impact Factor =N/A; Times Cited = 5.

10. “Conductance-Based Biophysical Distinction and Microfluidic Enrichment of Nanovesicles Derived from Pancreatic Tumor Cells of Varying Invasiveness”, J. H. Moore, W. B. Varhue, Y.-H. Su, S. S. Linton, V. Farmehini, T. E. Fox, G. L. Matters, M. Kester, N. S. Swami*, *Analytical Chemistry* (2019) 91, 10424-10431. Journal Impact Factor =6.5; Times Cited = 20.

11. “Electrofabricated biomaterial-based capacitor on nanoporous gold for enhanced redox amplification”, Y. Liu^{+}, J. McGrath^{+}, J. H. Moore, G. L. Kolling, J. A. Papin, N. S. Swami*, *Electrochimica Acta* (2019), 318, 828-836. Journal Impact Factor =5.4; Times Cited = 7.

12. Nanofluidic fluorescence microscopy with integrated concentration gradient generation for one-shot parallel kinetic assays, P Teerapanich, M Pugniere, C Henriquet, YL Lin, A Naillon, P Joseph, C.-F. Chou, T. Leichle, *Sensors and Actuators B: Chemical* (2018) 274, 338-342

3.3. Patent Applications

1. “Multiplexed control of impedance cytometry” (PCT/US2019/053242)

2. “Real-time control of acoustic resonance frequency variations in microfluidic devices” (U.S. Provisional Application 63/261,133)