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PNNL-36854

# Chemical Imaging for In Situ Detection and Discrimination of Aquatic Toxins Targeting Voltage Gated Sodium Channels

September 2024

Jennifer Mobberley
Jiyoung Son
Kristin Engbrecht

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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# Executive Summary

Biologically derived neurotoxins from cyanobacteria and algae impact environmental resources in addition to being considered a potential biological threat to human and animal health. Activity-based assays are essential to detecting and responding to toxic neurotoxin events either naturally occurring or deliberate. Two toxins of interest include saxitoxin and brevetoxin. These toxins bind to and alter the function of voltage-gated sodium channels (NaV channels) which are essential for generating cell membrane action potential. We report the development and refinement of a System for Analysis at Liquid Vacuum Interface (SALVI) to assess the functional activity of saxitoxin and brevetoxin. This approach utilizes a vacuum-compatible microfluidic reactor that permits analysis at the liquid vacuum interface of human derived cells with a neurotoxin of interest in a biologically relevant environment.

SALI based detection is technically relevant because of the following:

- Compatible with multiple detection modalities for real-time monitoring
  - Optical imaging techniques
  - Mass spectrometry characterization via ToF-SIMS

- Flexible platform that can be modified:
  - Numerous biological tissues can be integrated into the system
  - Types of marine toxin to be evaluated

This LDRD effort improved technical methods resulting in a reproducible mammalian cell cultivation and neurotoxin exposure system to detect differences of chemical signatures within the SALVI system. Additionally, this effort resulted in the following capability development:

- This project involved cross directorate collaborations (NSD and EBSD) to build cross-disciplinary capabilities that did not previously exist at PNNL. This involved cross-directorate networking and expanding microfluidics capabilities for national security research in Chemical and Biological Science group. The project also utilized the ToF-SIMS in EMSL for all measurements.

- Strategic support of multi-directorate PNNL staff members and refined mechanical and instrument operation analysis with ToF-SIMS.

- Methodology for the improved development of a SALVI prototype and assay to detect chemical biomarkers of neurotoxins in aquatic environments with a non-commercial system that offers flexibility for future efforts.

Future efforts will enable improved SALVI protocol development for multiple human tissue types and aquatic toxins. Additionally, multiple detection modalities could be implemented for enhanced confidence across an array of biological threat characterization measurements.

# Acknowledgments

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The authors would like to thank Dr. Xiao Ying Yu at Oak Ridge National Laboratory for her consultation on the ToF-SIMS work and Dr. Janine Hutchison at Pacific Northwest National Laboratory for her assistance with the cell cultivation capability.

# Acronyms and Abbreviations

| | |
|---|---|
| CDC | Center for Disease Control |
| HPLC | High Performance Liquid Chromatography |
| IC50 | Half Minimal Inhibitory Concentration |
| LC-MS/MS | Liquid Chromatography-Tandem Mass Spectrometry |
| LDRD | Laboratory Directed Research and Development |
| *m/z* | Mass to Charge Ratio |
| NaV | Sodium Voltage-gated channels |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| SALVI | System for Analysis at Liquid Vacuum Interface |
| SiN | Silicon Nitride |
| SME | Subject matter expert |
| ToF-SIM | Time-of-Flight Secondary Ion Mass Spectrometry |

# Contents

# 1.0 Background

Neurotoxins produced by aquatic microorganisms, such as cyanobacteria and algae, are an emerging biodefense, national security, as well as an economic challenge due to harmful impacts to humans, wildlife, and domestic animals. There has been a global increase and expansion of harmful algal blooms, events where neurotoxin-producing algae and cyanobacteria grow out of control, in fresh and marine waters.[1] Neurotoxins are potential biothreat agents. Specifically, saxitoxin is listed on the CDC select agent list and is covered under the Biological Weapons Convention, thus being able to detect them is important to national security.

A subset of these low molecular weight neurotoxins, including saxitoxin and brevetoxin, bind to and alter the function of voltage-gated sodium channels (NaV channels) which are essential for generating cell membrane action potential.[2] These cell membrane targets are pertinent for numerous biological tissues. The hundreds of known neurotoxins impacting sodium channels fall into two broad activity categories; pore-blockers, which plug the sodium channel (e.g., saxitoxin), and gating modulators (e.g., brevetoxin), which interfere with voltage-sensing of the channel. While there are several approaches to either categorizing neurotoxins or assessing their activities, they are either too specific, such as PCR and antibody-based enzyme linked immunosorbent assays; require live animals like the mouse bioassay; and/or require time consuming and expensive sample preparation prior to analysis like LC-MS/MS and HPLC. Furthermore, many of the common methods employed today depend upon identifying toxins based on known sequences or known toxin structure and thus miss toxins that may be structurally different but have the same activity.

As toxicity starts at the molecular level, where toxin detection should be targeted, many neurotoxins require transport across the cell membrane which disrupt ion concentrations across the membrane. Cell-based biosensors, which incorporate live cells for detecting neurotoxin threats, provide a way to observe and assess the mechanism of action of cytotoxicity on time scales of minutes to hours as opposed to waiting for complete cell or organism death.[3] It is critical these biosensors provide a way to observe biomarkers, which are measurable indicators of biological processes, in real-time. Cell-based biosensors also provide information that is needed to engineer biomaterial influenced designs that are more robust and fieldable.

PNNL has developed a System for Analysis at Liquid Vacuum Interface (SALVI) which is a vacuum-compatible microfluidic reactor that permits analysis at the liquid vacuum interface. SALVI fabrication involves using soft lithography to make a microchannel in a polydimethylsiloxane (PDMS) block that is then bonded to an electron transparent silicon nitride (SiN) window 100 nm thick suspended on Si chip substrate. The electron transparent SiN window enables a wide range of electron microscopy and other optical imaging techniques. The microchannel allows liquid to be flowed through the device, creating a microreactor that is amendable to in vitro or in situ cultivation and allows bioimaging of biological systems, including live cells.[4] Recently, C10 mouse alveolar type II lung cells were cultured in the microchannel and exposed to zinc oxide nanoparticle toxins.[5] This LDRD project built on this previous work to use SALVI's to examine sodium channel impacting neurotoxins on the membrane composition of cultivated human cells to develop biosensors for the monitoring of and detection of neurotoxins in aquatic environments.

## 1.1 Mission relevance

Multiple government agencies with responsibility for defense, security, and health are interested in robust and agent-agnostic approaches to biological threat detection as outlined in the National Biodefense Strategy. For toxin detection, current technologies are currently either not sensitive enough, can only detected a limited number of toxins, or require significant time and expensive resources. Due to the impact and necessity for accurate and sensitive detection of toxin threats of interests, our system will provide a framework for rapid assessment of toxin activity. SALVI microfluidics system provides a robust and tailored to detect pathogens (e.g., bacteria, yeast) or biotoxic chemicals (e.g., pharmaceutical based agents, nanoparticles) that impact the cell membrane.

## 1.2 Project objective and scientific approach

The objective of this project was to develop a cell-based microfluidic system using the PNNL developed System for Analysis at the Liquid Vacuum Interface (SALVI) for detection and identification of sodium-channel-impacting neurotoxin activity in aquatic environment. The central hypothesis was that active neurotoxins would elicit a measurable cell membrane response that differs from untreated samples within 4 hours of exposure enabling rapid threat identification. Figure 1 shows the scientific approach taken to meet this objective and test the hypothesis. The initial focus was on cultivation of our engineered cell line on silicon chips and refining or toxin exposure conditions for 'static ToF-SIMS' analysis using dried cell monolayers to have a semi-quantitative understanding of mass balance differences. The next step was to transition to performing liquid *in-situ* ToF-SIMS on live engineered cell line maintained in the SALVI. We anticipated that the liquid ToF-SIMS method would enable us to observe and dynamically monitor the surface cell membrane characteristics including lipids, proteins, and ion balance changes within the membrane. For detailed methods and materials, refer to appendix A.

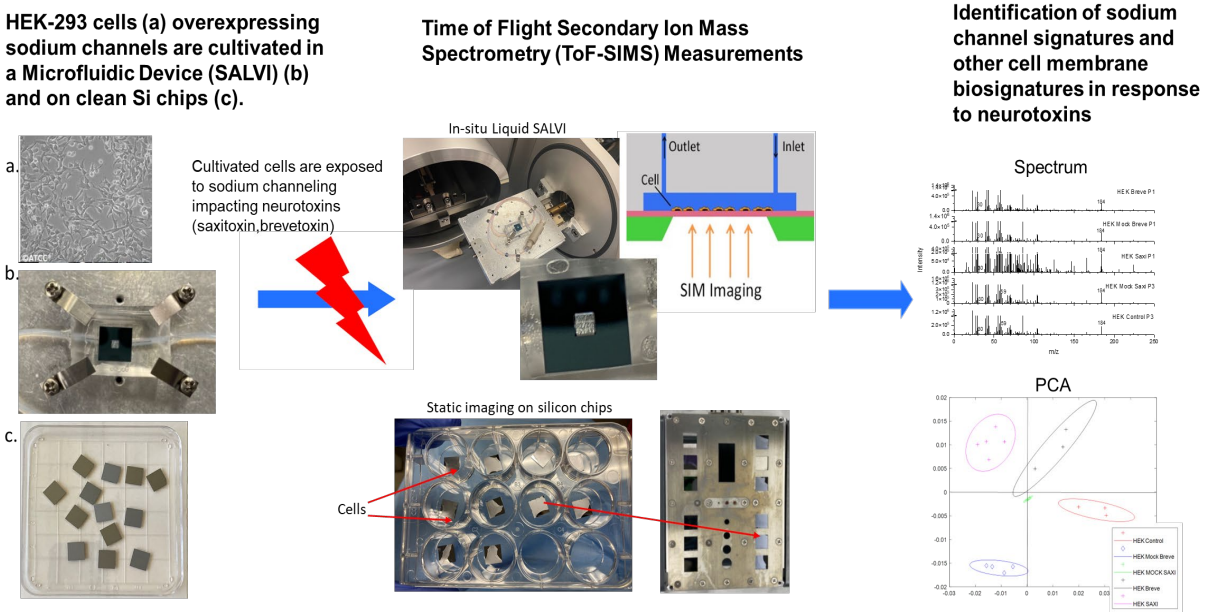


Figure 1. Overview of scientific approach for chemical imaging for in situ detection of neurotoxins with static ToF-SIMS with dried cells and liquid in-situ ToF-SIMS analysis with a System for Analysis at the Liquid Vacuum Interface (SALVI).

# 2.0 Results and Discussion

## 2.1 Protocol development for using the HEK-293 cell line for detecting neurotoxin activity with ToF-SIMS

The cell line selected for developing a prototype system was the human cell line HEK-293 (Human embryonic kidney 293 cells) that overexpresses the human SCN1A gene to create the isoform hNaV1.1 channel. This isoform is primarily found in the brain and other parts central nervous system and is a target of many sodium channel impacting neurotoxins including saxitoxin and brevetoxin.[7,8] An engineered HEK-293 cell line was chosen rather than a non-engineered cell line, such as Neuro-2a mouse neuroblastomas, as we were interested in having cells that had high numbers of our targeted sodium channel. The HEK-293 cell line stably transfected with the NaV1.1 channel were provided courtesy of Dr. Becky Hess (PNNL).

Our initial cultivation studies revealed that the HEK-293 cells were not able to consistently adhere to the silicon wafers and silicon nitride (SiN) windows for our static and in situ ToF-SIMS experiments, respectively. To aid with adherence, two coatings were tried: collagen and poly-L-lysine. While these coatings improved adherence of the cells, poly-L-lysine, a synthetic amino acid, performed the best and was selected for downstream experiments.

For toxin exposures, the concentrations of 10 nM of saxitoxin and 10 nM of brevetoxin were selected to be above the half-maximal inhibitory concentration (IC50) values reported for other engineered HEK-293 cells lines.[8,9] Due to time and experimental constraints, concentration range experiment were not performed in the current project. In developing exposure and chemical fixations protocol for the silicon wafers, it was observed that the cells would occasionally lift off the wafers even with the poly-L-lysine. This behavior was independent of the toxin presence and exposure time. Only those wafers that had cells attached to them were used for static ToF-SIMS analysis.

## 2.2 Static ToF-SIMS analysis to determine baseline biochemical signatures of sodium channel impacting neurotoxins

We performed 5 different experiments, referred to as sets, with toxin exposure for the static ToF-SIMS analysis. The initial 2 experiments were exploratory to test and refine our protocol development as such results are not included in this report. For each set there were six exposure treatments: cell only, cells exposed to saxitoxin or brevetoxin, and cells exposed to toxin solvent only, either 3 mM HCl (mock saxitoxin) or 50:50 ACN: water, 0.1% formic acid (mock brevetoxin). The mock samples control for any biochemical signatures that resulted from the solvent the toxin was in from the activity of the toxin itself. Following exposure, all samples underwent chemical fixation. One silicon wafer per treatment was analyzed on the TOF-SIMS.

Spectral principal component analysis (PCA) was performed to obtain a better understanding of cell molecular component differences among samples in the positive mode. All peaks in the mass-to-charge ratio (*m/z*) range of 20 – 800 were used in this analysis. The PCA score plot of the first two principal components (PCs) are depicted for three experimental sets in Figure 2A. The first two PCs explain between 87-92% of the variance observed between the different treatments within each experimental set. Across all three experiments, there is clear discrimination between the control and the treatments (toxin and mock) suggesting the treatments are impacting the normal functioning of the cells. There is also separation between the saxitoxin and brevetoxin samples and their respective mock treatments indicating that toxins impacted the cells differently

from the solvents found in the mock samples. The loading plots of PC1 and PC2 showed the key contributors to the observed similarities and differences among treatments (Figure 2B). The presence of the phospholipid phosphatidylcholine fragment (*m/z* 184) in all samples indicates that the spectra were collected from cells.

The initial analysis of the spectral peaks indicated that there were biochemical signatures indicative of blocking or modification of cellular voltage-gated sodium channels. While the sodium ion ($Na^+$, *m/z* 23) was present in all samples, the ion intensity was the highest in cells treated with saxitoxin. This suggests that saxitoxin actively blocked the hNaV1.1 channel during the exposure leading to increased sodium ion concentration in these cells. This increased ion intensity was not observed in the brevetoxin samples. Only the brevetoxin samples contained a peak *m/z* 39 which has identified as a cyclopropenyl fragment ($C_3H_3^+$). It's possible that this fragment is a result of breakdown of a high molecular weight fatty acid component of the cell membrane. Alternatively, this peak could be a potassium ion ($K^+$), but it's unclear why this would be elevated in these cells as brevetoxin does not impact potassium channels. The saxitoxin samples had several unique peaks or those with high counts compared to other treatments, including *m/z* 82 (cyclohexene, $C_6H_{10}^+$), 196 (hydrocarbon fragment, OH-$C_{11}H_{19}CO^+$), 266 (lipid fragment), and 288 (glycolipid fragment), which might be indicative of the oxidative stress due to toxin presence.[10] The brevetoxin samples also contained unique peaks and higher counts than other treatments, including *m/z* 262 (fragment of sphingomyelin) and 311 (fatty acid fragments), which also suggests oxidative stress is impacting the cells. ToF-SIMS analysis did not detect the presence of intact saxitoxin (*m/z* 229, $C_{10}H_{17}N_7O_4$) or brevetoxin (*m/z* 866, $C_{49}H_{70}O_{13}$). This is not unexpected given that these toxins only interact with the cell membrane and were likely washed away during chemical fixation.

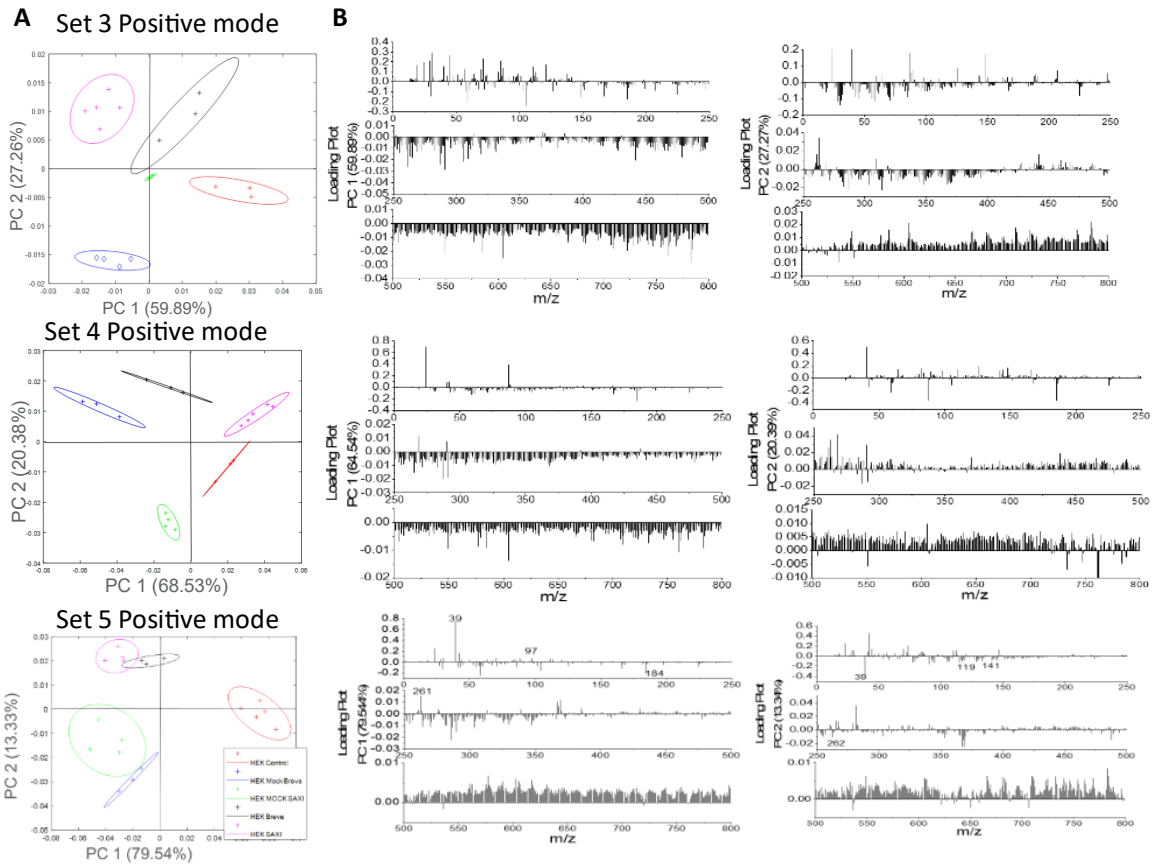


Figure 2. PCA results for three different experimental sets of ToF-SIMS data collected in positive mode. (A) The PCA score plot of the six treatments for each experimental set: cell only (HEK Control), cells exposed to saxitoxin (HEK Saxi) or brevetoxin (HEK Breve), and cells exposed to toxin solvent only either 3 mM HCl (HEK Mock Saxi) or 50:50 ACN: water, 0.1% formic acid (HEK Mock Breve). The ellipses denote the 95% confidence limit of each treatment is shown in the same color corresponding to each sample cluster. (B) The PC1 and PC2 loading plots show the spectra from 20 – 800 *m/z* responsible for the separation.

## 2.3 SALVI device for in situ ToF-SIMS on live cells

Following the work with static ToF-SIMS analysis, the toxin detection was transitioned to the microfluidic SALVI system. Optimization experiments involved determining the number of cells to seed into the SALVI system and the ideal channel size to achieve at least 70% cell coverage, minimize cells clogging the microfluidics components, and for the cells to remain adherent to the silicon nitrile window through the toxin exposure process. Initial experiments in 20 SALVI devices with a 200 micron channel seeded with 6-8 million cells formed a confluent monolayer of cells after 24-48 hours in 55% of the devices, but the movement and pressure of connecting the SALVI to a pump caused the cells to be washed away in around 90% of the SALVI devices tested. To mitigate these impacts, SALVI devices with 500 micron and 1 millimeter channels were tested with seeding 7-8 million cells followed by connection to the pump which resulted in 80% of the SALVI's having adherent cells. The 1 millimeter channels were chosen to be used for downstream

experiments because they had a higher percentage of adherent cells than the 500 micron channels. During all exposures of the liquid cultures to toxins and mock toxin samples, the cells immediately lifted off the SiN window in 100% of SALVI devices. Due to time constraints, only the cell and medium only SALVI devices underwent in situ TOF-SIMS imaging.

During transport from the laboratory to the instrument, loading the SALVI onto the stage and other ToF-SIMS preparations, some of the cells in the SALVI devices detached leading to only one cell only SALVI device being imaged. The in situ ToF-SIMS positive and negative spectra for media and cells only is shown in Figure 3. As observed in the static ToF-SIMS experiments, a phosphatidylcholine fragment peak (*m/z* 184, $C_5H_{15}NO_4P^+$) was only observed in the SALVI containing cells indicating this is a biomarker cells being present. There were also unique higher mass peaks in the media only (*m/z* 532) and cell only (*m/z* 628) suggesting unique larger molecules with positive charge, however these peaks remain unidentified.

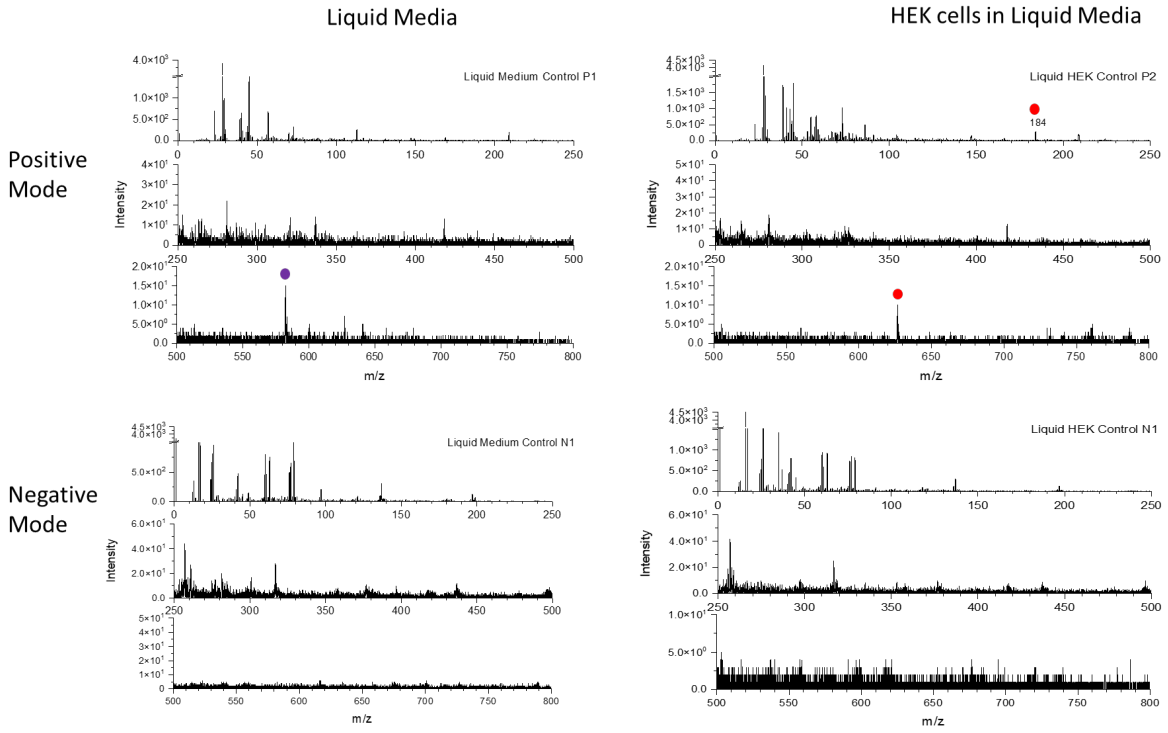


Figure 3. In situ ToF-SIMS of SALVI with liquid media or HEK cells. The data represents a single SALVI device for each treatment. The spectra from 20 – 800 *m/z* from both positive and negative mode are shown. The red dots indicate unique spectra found in HEK cell SALVI and purple dot indicate unique spectra found in liquid media.

# 3.0 Conclusions

## 3.1 Key Findings

- Established a reproducible cultivation and neurotoxin exposure protocol for HEK-293 cells overexpressing hNaV1.1 to analyze these samples with static ToF-SIMS.
- Detected differences in chemical signatures with static ToF-SIMS analysis between cells exposed to saxitoxin and those exposed to brevetoxin.
- Developed a SALVI system that is amenable to mammalian cell cultivation.

## 3.2 Key capabilities and products resulting from the project

- This project involved cross directorate collaborations (NSD and EBSD) to build cross-disciplinary capabilities that did not previously exist at PNNL. This involved cross-directorate networking and expanding microfluidics capabilities for national security research in Chemical and Biological Science group. The project also utilized the ToF-SIMS in EMSL for all measurements.
- The TOF-SIMS data from this project is a unique and novel dataset. To date, no publications utilizing TOF-SIMS to examine cellular response to marine neurotoxins have been published to date.
- Dr. Jiyoung Son gave an oral presentation “Chemical imaging for in situ detection and discrimination of aquatic toxins targeting voltage gated sodium channels (VGSC)” at the American Vacuum Society International Symposium and Exhibition in November 2023.

## 3.3 Lessons learned from technical challenges

The major goal of this project was to develop a prototype of the SALVI device that could be used for detecting chemical biomarkers of neurotoxins in aquatic environments. While this goal was not achieved, the project team has identified several technical challenges and limitations that need to be overcome to develop a prototype cell-based biosensor that can be used to monitor neurotoxin activity in aquatic environments.

This project attempted cultivation of mammalian cells in a non-commercial microfluidic system that had been developed for inorganic materials analysis and limited bacterial biofilm work. While a previous PNNL project successfully used the SALVI device to analyze a mammalian cell-line, that work had a well establish cell culturing process and nanoparticle treatment exposure protocol in place (Hu et al., 2016). For the current project, we had to acquire the HEK-293 cell-lines (these were not obtained until March 2023 due to technical issues) and develop cultivation and toxin exposure protocols that could be used in both static ToF-SIMS and in situ SALVI device. This process required several months of experimentation and troubleshooting to adapt this for a cell-line that ultimately was determined not to be ideal for the current microfluidic design of the SALVI. Some of the major engineering challenges that need to be overcome include identifying a cell line that is highly adherent, improving gas exchange and minimizing the impact of pressure changes during toxin exposures. The SALVI device should be redesigned with increased tubing width and channel size to minimize some of the impacts we observed in the current study.

An additional unexpected technical challenge was the loss of strategic support for ToF-SIMS before and towards the end of the project. The primary SALVI SME left the laboratory before the start of the project, leaving one technical expert to assist with the project. There was no training of additional staff due to time and budget constraints. This technical expert left PNNL before the end of the project leading to unfinished final analyses. In addition, the ToF-SIMS instrument in BSF which was anticipated to be the instrument used for the project was decommissioned prior to the beginning of ToF-SIMS work. While we were able to use the ToF-SIMS at EMSL under "contracted time proposal", the use of this core instrument impacted the project in several ways including having to schedule our experiments around other users with higher priority and being unable to independently operate and collect data which limited the amount of protocol development that could be done.

## 3.4 Next steps

- While initial analysis of the static SIMS data sets has been completed a more in-depth analysis of the spectral peak identifications across the three experimental sets needs to be explored.

- Develop an improved SALVI system for liquid ToF-SIMS analysis of cell signatures associated with neurotoxin activity. This would require the exploration of additional cells line that are more adherent, engineer the microfluidics system to be compatible with mammalian cells, and optimize toxin exposure (concentration and time) for the SALVI. Additional chemical imaging capabilities at PNNL could also be leveraged to explore cell signatures.

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# Appendix A – Materials and Methods

## A.1 Cell Culture

For this project, human embryonic kidney cells (HEK-293) that overexpress the SCN1A gene (encodes the alpha subunit of the sodium channel 1.1) were used for the static and liquid SALVI experiments. HEK-293 cells were maintained in T150 flasks (Corning) in Dulbecco's modified eagle's medium (DMEM, Gibco) cell culture medium, supplemented with 1% glutamax (Gibco), 1% antibiotic/antimycotic (Gibco), 10% fetal bovine serum (Gibco), and 10 µg/mL puromycin (Fisher Scientific). Cells were incubated in a humidified atmosphere at 5% $CO_2$ at 37 °C. Cells were passaged once confluent using phosphate-buffered saline (Gibco) and 0.25% trypsin-EDTA (ThermoFisher Scientific). The incubation times ranged between 1 to 2 hours depending on the state of the cells.

## A.2 Toxin Exposure

Toxin exposure studies examined the impact of two different commercially available neurotoxins: a blocking toxin, saxitoxin (National Research Council Canada), and a modulating toxin, brevetoxin (Abcam). Saxitoxin was resuspended in 3 mM HCL and brevetoxin was resuspended in 50:50 ACN: water, 0.1% formic acid. For static and liquid SALVI experiments, the cells were exposed to several treatments: 10 nM of either saxitoxin or brevetoxin, 3 mM HCL in cell culture medium (mock saxitoxin), 50:50 ACN: water, 0.1% formic acid in cell culture medium (mock brevetoxin), or just cell culture medium.

## A.3 Static ToF-SIMS

Static ToF-SIMS was performed by analyzing dried cell monolayers on silicon nitride (SiN) membranes. For the dried sample preparation, HEK-293 cells were seeded at a density of 5 x $10^5$ cells per mL onto individual, poly-L-lysine (Millipore) coated SiN membranes. As a control, we also had poly-L-lysine coated SiN membranes that received no cells. 24 hours later, the culture medium was removed and 2 mL of each of the treatments (listed above in section A.2) were added to select SiN membranes. After incubation, all of the SiN membranes (with and without cells) were chemically fixed. To remove the salts on the cells, all of the membranes were washed with 150 mM ammonium acetate (Sigma), incubated and fixed in 4% paraformaldyhyde (Sigma) solution for 30 minutes, and then rinsed with cell culture grade water (Corning). Finally, the membranes were allowed to air dry and were then stored at 4 °C until static ToF-SIMS analysis could be performed.

A ToF-SIMS 5 spectrometer (IONTOF, GmbH, Münster, Germany) was used to collect the data. To better capture organic components, a pulsed 25 KeV Bi3+ primary ion beam was used to collect the data. The analysis beam scanned over a ~ 500 µm diameter (128 pixels). There were 60 scans collected with 5 data points in each mode, positive and negative, on each sample for a total of 10 data points.

## A.4 Fabrication of SALVI devices

The PNNL developed System for Analysis at Liquid Vacuum Interface (SALVI) is a vacuum-compatible microfluidic reactor that permits analysis at the liquid vacuum interface.[4] SALVI fabrication involves using soft lithography to make a microchannel in a polydimethylsiloxane (PDMS) block that is then bonded to a 100 nM thick electron transparent silicon nitride (SiN) window suspended on Si chip substrate. Several different microchannels widths were tested but 1mm for cells were selected and 200 uM for medium only.

## A.5 Liquid ToF-SIMS

A single SALVI was used for each experiment. The microchannel in each SALVI was rinsed with 70% ethanol and cell culture water, incubated with poly-L-lysine for 5 minutes, and then rinsed with cell culture water and cell culture medium. A HEK-293 cell suspension at 8 x $10^6$ cells per mL was then injected into the channel and incubated in a humidified atmosphere at 5% CO2 at 37°C for 48 hours. Fresh cell culture medium containing each of the treatment groups described in section A.2 were pumped through the channels at 3uL/minute. The SALVI devices were immediately analyzed on liquid ToF-SIMS.

A ToF-SIMS 5 spectrometer (IONTOF, GmbH, Münster, Germany) was used to collect the data. To better capture organic components, a pulsed 25 KeV Bi3+ primary ion beam was used to collect the data. The analysis beam scanned over a ~ 2 µm diameter (64 pixels). There were a total of 1160 scans collected with 4 data points in each mode, positive and negative, for a total of 8 data points per sample. After the SiN window was penetrated an additional 100 scans were collected.

## A.6 Analysis of ToF-SIMS

All data were analyzed using the IONTOF Surface Lab software (version 7.0). Mass spectra were calibrated using $CH^{3+}$, $C_3H_4NO^+$, $C_8H_8NO^{3+}$ in positive spectra and $CH_2^-$, $C_3H_2N_2^-$, $C_5H_4O_5^-$ in negative spectra. For spectral principal component analysis, the mass calibrated data were exported to Origin Pro (2019b) for analysis.

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