LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-TR-739181

A Portable Cell Maintenance System for Rapid Toxicity Monitoring Final Report CRADA No. TC-02081-04

S. Kane, P. Zhou

September 28, 2017

Disclaimer

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

A Portable Cell Maintenance System for Rapid Toxicity Monitoring

Final Report
CRADA No. TC-02081-04
Date Technical Work Ended: November 17, 2006

Date: November 21, 2006 Revision: 0

A. Parties

This project was a relationship between Lawrence Livermore National Laboratory (LLNL) and Kionix, Inc.

The Regents of the University of California
Lawrence Livermore National Laboratory
7000 East Avenue
Livermore, CA 94550
Staci Kane, Ph.D., L-542
Tel: (925) 422-7897
Fax: (925) 422-3800

Kionix, Inc.
36 Thornwood Drive
Ithaca, NY 14850
Peng Zhou, Ph.D.
Tel: (607) 257-1080
Fax: (607) 257-1146

B. Project Scope

The Phase I STTR research project was targeted at meeting the objectives and requirements stated in STTR solicitation A04-T028 for a Portable Cell Maintenance System for Rapid Toxicity Monitoring. In accordance with the requirements for STTR programs, collaboration was formed between a small business, Kionix, Inc., and The Regents of the University of California, Lawrence Livermore National Laboratory (LLNL). The collaboration included CytoDiscovery, Inc. (CDI) which, in collaboration with Kionix, provided access to membrane chip technology and provided program support and coordination. The objective of the overall program (excerpted from the original solicitation) was: "To develop a small, portable cell maintenance system for the transport, storage, and monitoring of viable vertebrate cells and tissues." The goal of the Phase I project was to demonstrate the feasibility of achieving the program objectives utilizing a system comprised of a small-size, microfluidic chip-based cell maintenance cartridge (CMC) and a portable cell maintenance system (CMS) capable of housing a minimum of four CMCs. The system was designed to be

capable of optimally maintaining multiple vertebrate cell types while supporting a wide variety of cellular assays.

This project consisted of the following major tasks and deliverables:

Task 1: Design, fabricate, and demonstrate a microfluidic chip based CMC capable of maintaining cells in optimal condition that is consistent with transport/storage in the CMS and compatible with optical and electrical assay readouts in a portable detection system. The duration of this task was 11 months. (Kionix and LLNL)

Task 2: Demonstrate on-chip viability, vitality and function with CHO and human primary fibroblast cells for three (3) days. The duration of this task was 11 months. (LLNL)

Task 3: Demonstrate the feasibility of on-chip pH and osmolarity compensation to reduce the possibility of false negatives/positives from environmental differences between the cell culture medium and the sample. The duration of this task was 8 months. (Kionix and LLNL)

Task 4: Compile a product requirements document for the CMS and CMC as the output of Phase I, and write a Phase II proposal. The duration of this task was 8 months. (Kionix)

Deliverables:

1. Prototype chips and fluid delivery system delivered by Kionix to LLNL for the purpose of achieving Tasks 2 and 3. CMC design will be optimized to reproducibly, maintain cells in healthy condition for up to three days storage in the chip environment. Due within the first month after CRADA was executed chips were supplied by Kionix as needed by LLNL throughout the twelve (12)-month funding period. (Kionix/LLNL)

2. Summary of experiments to evaluate viability, vitality and function of cells in chip with different design and testing variables. Due at completion of Phase I. (LLNL)

3. Summary of initial findings regarding on-chip pH and osmolarity compensation and/or requirements for compensation. Due at the completion of Phase I. (Kionix)

4. Product requirements document for the CMS and CMC. Due within 30 days after successful completion Phase I. (Kionix)

5. Final Report and Abstract due within 30 days of completion or termination of the project, as required under Article XI of the CRADA. (LLNL/Kionix)

This project was originally designated as a twelve (12) month project. Two No-Cost Time Extension Requests were executed for this CRADA, extending the expiration date of this project to November 17, 2006. All of the above tasks and deliverables were successfully completed as part of the Phase I STTR project. Monthly reports and a Final Phase I project report were completed on time. In addition, a Phase II STTR proposal was submitted. The proposal

received quite positive feedback, although it was not funded. It should be noted that no Phase II proposals were supported under the solicitation for a cell maintenance system for water toxicity testing.

C. Technical Accomplishments

The primary goals of this project have been successfully achieved. A cell maintenance cartridge (CMC) has been developed and demonstrated which is capable of maintaining cell viability for at least three days with multiple cell types. The CMC developed in this project represents a significant technological breakthrough toward the realization of a functional, practical, and commercially viable method for widespread deployment of cellular assays into field settings that was heretofore not possible without a localized cell culture facility.

The specific technical accomplishments of this CRADA are summarized below in terms of results from cellular assays conducted with different CMC designs, development of CMC and membrane chips, and operational testing of the system for using the CMC for water toxicity testing.

The following table summarizes the results of the cellular assays carried out under this CRADA.

Cellular Assays:

Assays:	Viability	Vitality	Proliferation	Function
Cell lines:				
CHO	>3 days (37C & RT)	>3days (37C & RT)	5days	
Engineered CHO (GFP expressing)	>3 days	>3days	>3days	>3 day
WI 38 human fibroblast (neuronal size)	6 day	6 day		

CHO = Chinese Hamster Ovary; GFP = Green Fluorescent Protein; WI38 is a human fibroblast primary cell line.

For all three of the cell lines tested, viability and vitality was demonstrated for at least three days thus meeting the primary objective of Phase I. Furthermore, for WI38 cells, viability and vitality were demonstrated for up to six days.

CMC:

Sterility	Bacteria barriers installed on each well to provided sterile operation. No bacterial contamination was observed.
Flow control system	Embedded flow control system based on differential evaporation. It was shown that there were no deleterious effects from salt build up in output well or due to back diffusion of waste products from the output well into the assay channels.

As the table above indicates, the CMC was designed with bacteria barriers covering self-contained media and waste reservoirs. Flow was generated and maintained by the use of an aperture plate, which provided small and large apertures over the media and waste wells, respectively. The differential apertures produced a differential rate of evaporation in the inlet and

outlet wells and thus a continuous flow from the media supply well was initiated from the inlet well to the outlet well to replace what was lost due to evaporation. Electrical conductivity measurements showed that salt build-up in the output wells was not significant during the experimental time frame and diffusion experiments indicated that the flow rate in channels was large enough to overcome back diffusion of salt or cellular waste products into the channels. We thus concluded that there were no negative effects on the cells in the channels due to build up of salt in the output wells.

Membrane Chip:

Evaluated standard and deep channel designs	Successfully loaded small and large cell types into standard (depth =30μm) and deep channels (depth = 80μm)
Standard well design	3 day limit discovered for fast growing CHO cells due to channel clogging
Evaluated alternative well designs	Bead experiment showed that multi-input wells reduce cell density at channel inlets and should significantly reduce channel clogging due to cell overgrowth

The most significant limitations to viability and proliferation were associated with cell overgrowth in the wells or channels with rapidly dividing cells (e.g., CHO cells), the need for periodic replenishment of the CMC's self contained media reservoir, and a need to improve the consistency for successful loading and seeding of cells. Several strategies have been identified for dealing with each of theses issues including but not limited to: optimizing the manufacturing process and the design and dimensions of the media supply wells and /or channels, reducing the cell seeding density, reducing the storage temperature, and adjusting the media composition or incubation environment variables such as CO_2 concentration. For slow or non-growing cells like fibroblasts, neurons, or lymphocytes, overgrowth is not expected to be a problem.

System:

pH and osmolarity of water samples	Confirmed that expected pH and osmolarity range extremes do not effect cells when added to lyophilized media
Osmolarity monitoring in CMS for QC purposes	Electrical conductivity has been reported to be an acceptable surrogate for osmolarity in biological fluids where the osmolarity is dominated by ionic species [4, 5].

We hypothesized that drinking water samples to be tested could first be filtered to remove particulates and bacteria and then added to either lyophilized or concentrated cell culture media prior to introduction into the chip. We demonstrated that this method would not create artifacts due to potential extremes in either the pH or the osmolarity of the water samples. This was accomplished by combining lyophilized cell culture media with water samples having values of pH (between 6 and 8.5) and osmolarity (3g/L total dissolved solids) representing values

exceeding the literature reported extremes for drinking water [1,2,3]. Since no deleterious effects were observed, relative to controls, using media formulated from samples at the extremes, we believe that our hypothesis was confirmed. Therefore, we will assume that in actual usage of the CMC, water samples will be pre-filtered, mixed with either lyophilized or concentrated cell culture media and then introduced into the chip.

It has been reported that osmolarity adjustment is necessary for certain biosensor cell lines [6] when the osmolarity of the water sample is significantly lower than that of the control media. However, using our method of adding the sample to lyophilized media, the osmolarity would be increased (not decreased) above the level of the control media by an amount determined by the osmolarity of the water sample. Given the literature reported osmolarity range extremes, increases in osmolarity would be expected to be slight to moderate. As mentioned above, we observed no effect on the cell types we investigated even with moderate changes in osmolarity. However, the possible effects of osmolarity changes on candidate biosensor cell lines and methods of compensation was beyond the scope of the Phase I investigation and it is expected that each cell line would need to be individually characterized on a case be case basis.

In summary, the CMC (which met the Phase I STTR requirements) is about the size of a microscope slide and is compatible with operation in standard microscopes or with portable reading devices to be developed in the future. The CMC is highly reliable and contains no moving parts. Its design is compatible with injection plastic molding so that the CMC could be implemented as a low cost disposable cartridge when produced in production quantities. The use of micro-scale fluidics (via the membrane chip) dramatically reduced the size of fluid reservoirs required to sustain cell cultures for extended time periods, while maintaining a small size, low cost disposable cartridge.

An inherent feature of our gas permeable membrane chip is that the cells housed within the channels are in diffusive communication with the incubator environment and therefore are maintained under ideal conditions. Our evaporation controlled fluid delivery system is easily scalable to larger reservoir volumes. Initial calculations indicate that 2-4 weeks of cell viability are readily achievable. Furthermore, our fluidic system is compatible with supplemental fluid delivery systems, which could further extend the capabilities of the CMC if needed in the future.

We have also demonstrated through the use of Kionix rapid prototyping capability that membrane chips with custom channel and well dimensions can be rapidly developed and optimized for new cell types. Furthermore, we are confident that our rapid prototyping capabilities can be employed to further modify the CMC to meet the optical or electrical readout requirements of a wide range of engineered biosensor cell types.

Several Phase I requirements were actually exceeded by a considerable margin. Cell viability for WI38 human fibroblasts was actually demonstrated for 6 days rather than 3 as originally specified. The performance of our evaporation controlled fluid delivery system has exceeded our expectations and has the potential to yield a simple, reliable, and low cost injection molded disposable cartridge when implemented for production quantities.

Other than the problem of cell overgrowth that was observed with the rapidly growing CHO cells that were tested, there has been no fundamental limit identified that would restrict the time that cells could be maintained within the CMC (the Phase II target was specified as 14 days).

The problem of overgrowth can be readily addressed using strategies including but not limited to: optimizing the design and dimension of the chip wells and /or channels, reducing the cell seeding density, reducing the storage temperature, and adjusting the media composition or incubation environment variables such as CO_2 concentration. For slow or non-growing cells like fibroblasts, neurons, or lymphocytes, overgrowth is not expected to be a problem.

We have proposed and demonstrated a simple and practical sample preparation method that ameliorates the problem of pH and osmolarity differences between water samples and cell culture medium (controls). Our experiments have shown the feasibility of a sample preparation method in which the water sample is first filtered to remove particulate matter and then added to either lyophilized or concentrated cell culture media prior to injection into the CMC. The osmolarity and buffering capacity of cell culture media are sufficiently high so as to dominate over the reported extremes for potable water. We have used the assays and techniques developed in Phase I to demonstrate that our sample preparation method can be successfully employed with water samples having pH and osmolarity values at or beyond the literature reported extremes.

D. Expected Economic Impact

The key goal of this project was to develop a standardized platform to enable the development and deployment of a panel of accurate, reliable, and cost-effective cellular assays with broad based and accurate contamination detection and attribution capabilities. The commercial motivation is consistent with the generally perceived need for an accurate and cost–effective method of assuring the safety of drinking water, i.e., “lack of contamination” in military, civilian, and industrial settings. For example, in the United States alone, there are about 54,000 community water systems and approximately 500 of these systems serve more than 100,000 people each. The fact that about 3,800 of these larger water systems service over 80% of the US population means that large numbers of people are at risk from the effects of contamination if a single water supply were to be contaminated. For example, malicious contamination by a potent toxin of the water supply for a single large building in an urban area could potentially jeopardize the lives of thousands of people.

In addition to the military, prospective customers could be expected to be the water utilities as well as researchers as in academia and governmental agencies such as the US EPA, CDC, USGS, and the DOE and DOD National Laboratories. Assuming widespread use of the technology comparable for example, to the number of fecal coliform assays currently performed in urban drinking water supply screening, utilization could exceed 500,000 tests per year at an average cost of $50-$100 per test chip (panel of tests) or about $25-50 million dollars per year.

D.1 Specific Benefits

Benefits to DOE

The benefit to DOE will be in providing a research and evaluation service for the development of this microfluidic technology that is consistent with its mission in environmental monitoring and stewardship. Findings will be highly visible and will provide a framework for successfully

competing for related proposal calls potentially resulting in additional funding and future royalties through patents and licenses.

Benefits to Industry

The CRADA project was to develop a cell maintenance cartridge and system (CMC/CMS) to keep vertebrate cells in optimal condition to enable high quality assays focused on water monitoring for toxic contaminants. The technology development will benefit agencies involved in maintaining safe and secure drinking water supplies and therefore, the results will benefit human health in the civilian and military sectors. The benefit to Kionix will be that it can license the technology and / or market chips for conducting cell-based assays for water monitoring, in addition to other potential markets/applications in the pharmaceutical industry.

E. Partner Contribution

Kionix was tasked with the design, fabrication and modification and physical testing required to develop a microfluidic chip based CMC capable of maintaining cells in optimal condition consistent with transport/storage in the CMS and compatible with optical and electrical assay readouts in a portable detection system. Kionix conducted testing on chips for media supply, in terms of flow rates and influence on accumulation of media salts. Kionix supplied chips and accessories to LLNL for conducting research using human primary and cultured cell lines. They also conducted training for LLNL personnel for chip loading, handling and analysis, and provided support for technical issues throughout the project.

The core components of the CMC developed by Kionix consisted of a plastic chip with 8 independent embedded microfluidic cellular assay channels covered by a gas permeable membrane, which ensured sufficient diffusive communication of oxygen and carbon dioxide between the cells on the chip and the environment of the CMS (Figure 1). The design of the chip allowed for 8 different assays to be run simultaneously per CMC. The development included modifications such a cover plate with micro-porous frits to provide for evaporation driven flow control and bacteria barriers to keep the internal space of the CMC sterile, a reagent manifold to provide storage spaces for cell medium or other buffer/reagent and a stainless steel clamp to keep all of the components together.

Kionix (in collaboration with CytoDiscovery) considered various fluid delivery and control strategies for the CMS and CMC modules, with the goal of meeting the requirements for storage and transport under the cell culture conditions. Throughout the CMC testing period, Kionix made modifications to the chip channel and manifold dimensions (i.e., inlet well volume) to accommodate the needs for the different cell lines and in order to optimize cell culture conditions in the chip. Kionix investigated gravity driven microfluidic flow as a scalable and simple means to generate simultaneous parallel nl/min to μl/min flow rates through multiple channels. In order to create a long lasting gravity induced flow within the CMC, Kionix developed a novel cover plate (Figure 1), which generates *controlled differential evaporation* between the input and outlet reagent wells. This cover plate comprises no moving parts and requires no physical interfaces to external sources of energy or fluid. Kionix performed testing to ensure that chip operational parameters (e.g., flow rate, media salt accumulation) were within a sufficient range for optimal cell maintenance.

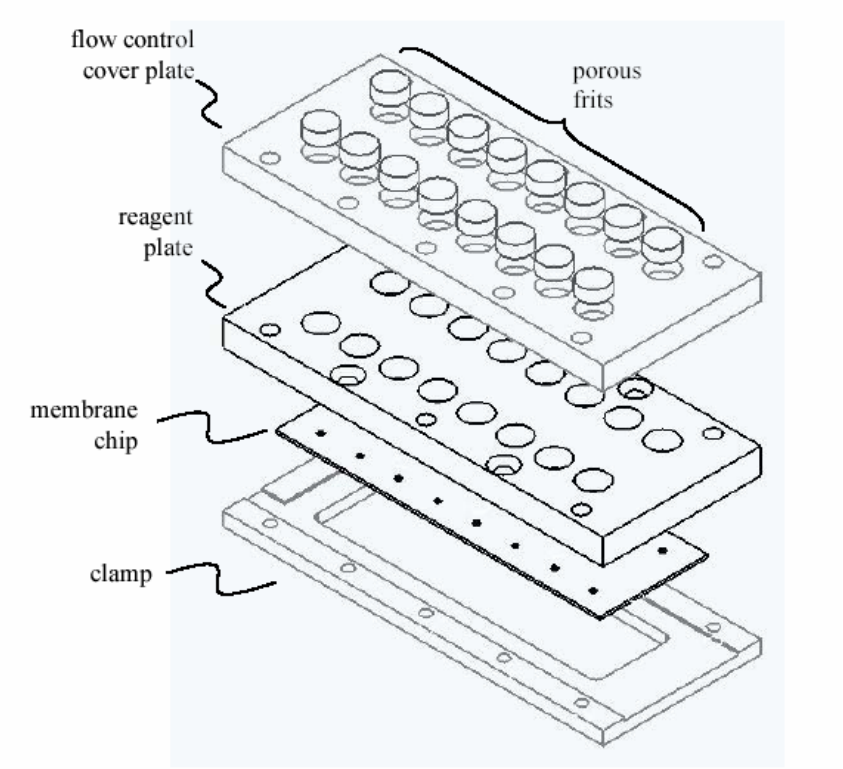

Figure 1. Exploded view of the CMC developed during the Phase I STTR project.

F. Documents/Reference List

Reports

Monthly reports (six total) and a final report were generated and submitted to the Army STTR program office by Kionix with input from LLNL and CytoDiscovery.

Copyright Activity

None.

Subject Inventions

None.

Background Intellectual Property

LLNL did not disclose any Background Intellectual Property (BIP) for this CRADA.

Kionix disclosed the following BIP for this CRADA:

U.S. Patent Application No. PCT/US03/40107; Filed: 12/16/03; "Microfluidic System with Integrated Permeable Membrane"; Inventors: Douglas N. Modlin and David Chazan

U.S. Provisional Patent Application No. 60/562,594, Filed: April 14, 2004; "A Microfluidic System for Rapid Toxicity Monitoring"; Inventors: Douglas N. Modlin, Peng Zhou, and Lincoln Young

References

[1] http://www.water-research.net/ph.htm

[2] http://www.epa.gov/safewater/consumer/2ndstandards.html

[3] http://www.epa.gov/safewater/dwa/electronic/presentations/uic/pt2/uic31.html

[4] Fouke, J. M.; Wolin, A. D.; Saunders, K. G.; Neuman, M. R.; McFadden, E. R., Jr. IEEE Trans. Biomed. Eng. 1988,35, 877-881.

[5] Mitsubayashi, K.; Yokoyama, K .;Toshifumi, T.; Tamiya, E.; Isao Rarube,I., Anal. Chem. 1993, 65, 3586-3590

[6] Mourlas, N.J. et al. 2002. An in-line osmometer for application to a cell-based biosensor system. Sensors and Actuators B. 83: 41-47.

G. Acknowledgement

Industrial Participant's signature of the final report indicates the following:

1) The Participant has reviewed the final report and concurs with the statements made therein.
2) The Participant agrees that any modifications or changes from the initial proposal were discussed and agreed to during the term of the project.
3) The Participant certifies that all reports either completed or in process are listed and all subject inventions and the associated intellectual property protection measures generated by his/her respective company and attributable to the project have been disclosed and included in Section E or are included on a list attached to this report.
4) The Participant certifies that if tangible personal property was exchanged during the agreement, all has either been returned to the initial custodian or transferred permanently.
5) The Participant certifies that proprietary information has been returned or destroyed by LLNL.

P Zhou 2/27/07

Peng Zhou, Ph.D.
Principal Investigator
Kionix, Inc.

Date

Staci Kane 3/2/07

Staci Kane, Ph.D.
Principal Investigator
Lawrence Livermore National Laboratory

Date

Karena McKinley 3/27/07

Karena D. McKinley, IPAC Director
Lawrence Livermore National Laboratory

Date

Attachment I – Final Abstract

A Portable Cell Maintenance System for Rapid Toxicity Monitoring

Final Abstract (Attachment I)
CRADA No. TC-02081-04
Date Technical Work Ended:

Date: November 21, 2006 Revision: 0

A. Parties

This project was a relationship between Lawrence Livermore National Laboratory (LLNL) and Kionix, Inc.

The Regents of the University of California
Lawrence Livermore National Laboratory
7000 East Avenue
Livermore, CA 94550
Staci Kane, Ph.D., L-542
Tel: (925) 422-7897
Fax: (925) 422-3800

Kionix, Inc.
36 Thornwood Drive
Ithaca, NY 14850
Peng Zhou, Ph.D.
Tel: (607) 257-1080
Fax: (607) 257-1146

B. Purpose and Description

The CRADA between Kionix, Inc., and The Regents of the University of California, Lawrence Livermore National Laboratory (LLNL) centered on a Phase I STTR research project for development of a Portable Cell Maintenance System for Rapid Toxicity Monitoring, which was of interest to the Army STTR program office. The collaboration included CytoDiscovery, Inc. (CDI) which, in collaboration with Kionix, provided access to membrane chip technology and provided program support and coordination. The objective of the overall program (excerpted from the original solicitation) was: "To develop a small, portable cell maintenance system for the transport, storage, and monitoring of viable vertebrate cells and tissues." The goal of the Phase I project was to demonstrate the feasibility of achieving the program objectives utilizing a system comprised of a small-size, microfluidic chip-based cell maintenance cartridge (CMC) and a portable cell maintenance system (CMS) capable of housing a minimum of four CMCs. The system was designed to be capable of optimally maintaining multiple vertebrate cell types while supporting a wide variety of cellular assays.

This project consisted of the following major tasks and deliverables:

Task 1: Design, fabricate, and demonstrate a microfluidic chip based CMC capable of maintaining cells in optimal condition that is consistent with transport/storage in the CMS and compatible with optical and electrical assay readouts in a portable detection system.
Task 2: Demonstrate on-chip viability, vitality and function with CHO and human primary fibroblast cells for three (3) days.
Task 3: Demonstrate the feasibility of on-chip pH and osmolarity compensation to reduce the possibility of false negatives/positives from environmental differences between the cell culture medium and the sample.
Task 4: Compile a product requirements document for the CMS and CMC as the output of Phase I, and write a Phase II proposal.

Deliverables:

1. Prototype chips and fluid delivery system delivered by Kionix to LLNL for the purpose of achieving Tasks 2 and 3. CMC design was optimized to reproducibly, maintain cells in healthy condition for up to three days storage in the chip environment.
2. Summary of experiments to evaluate viability, vitality and function of cells in chip with different design and testing variables.
3. Summary of initial findings regarding pH and osmolarity requirements for compensation.
4. Product requirements document for the CMS and CMC.
5. Final Report and Abstract.

This project was originally designated as a twelve (12) month project. Two No-Cost Time Extension Requests were executed for this CRADA, extending the expiration date of this project to November 17, 2006. All of the above tasks and deliverables were successfully completed as part of the Phase I STTR project. Six monthly reports and a Final Phase I project report were completed on time.

A cell maintenance cartridge (CMC) was developed and demonstrated that was capable of maintaining cell viability for at least three days (and up to 6 days for one cell type) with multiple cell types. The CMC met all Phase I STTR requirements. The CMC was highly reliable, contained no moving parts, and could be implemented as a low cost disposable cartridge when produced in production quantities. The use of micro-scale fluidics (via the membrane chip) dramatically reduced the size of fluid reservoirs required to sustain cell cultures for extended time periods, while maintaining a small size, low cost disposable cartridge. The gas permeable membrane chip allowed diffusive communication with the incubator environment, such that cells were maintained under ideal conditions. The evaporation controlled fluid delivery system can easily be scaled to larger reservoir volumes, thus potentially allowing 2-4 weeks of cell maintenance. The membrane chips with custom channel and well dimensions can be rapidly developed and optimized by Kionix for new cell types.

Other than the problem of cell overgrowth that was observed with rapidly growing cells that were tested, there has been no fundamental limit identified that would restrict the time that cells could be maintained within the CMC (the Phase II target was specified as 14 days). Strategies to

address overgrowth may include optimizing the design and dimension of the chip wells and /or channels, reducing the cell seeding density, reducing the storage temperature, and adjusting the media composition or incubation environment variables such as CO_2 concentration. For slow or non-growing cells like fibroblasts, neurons, or lymphocytes, overgrowth is not expected to be a problem.

A simple and practical sample preparation method was demonstrated that ameliorated the problem of pH and osmolarity differences between water samples and cell culture medium (controls). The water sample was filtered to remove particulate matter and then added to either lyophilized cell culture media prior to injection into the CMC. The osmolarity and buffering capacity of cell culture media was sufficiently high so as to dominate over the reported extremes for potable water.

Based on successful completion of this project, a Phase II STTR proposal was submitted. While the proposal received quite positive feedback, it was not funded, and furthermore no Phase II proposals were supported under the solicitation for a cell maintenance system for water toxicity testing.

C. Benefit to Industry

The CRADA project was to develop a cell maintenance cartridge and system (CMC/CMS) to keep vertebrate cells in optimal condition to enable high quality assays focused on water monitoring for toxic contaminants. The technology development will benefit agencies involved in maintaining safe and secure drinking water supplies and therefore, the results will benefit human health in the civilian and military sectors. The benefit to Kionix will be that it can license the technology and / or market chips for conducting cell-based assays for water monitoring, in addition to other potential markets/applications in the pharmaceutical industry.

D. Benefit To DOE/LLNL

The benefit to DOE will be in providing a research and evaluation service for the development of this microfluidic technology that is consistent with its mission in environmental monitoring and stewardship. Findings will be highly visible and will provide a framework for successfully competing for related proposal calls potentially resulting in additional funding and future royalties through patents and licenses.

E. Project Dates

November 17, 2004 to November 17, 2006