DEPARTMENT OF HEALTH & HUMAN SERVICES — Public Health Service

National Institutes of Health
National Center for
Research Resources
Bethesda, Maryland 20892

Automatic Withdrawal Date: 03/31/03

APR 0 6 2002

Joseph D. Andrade, Ph.D.
Distinguished Professor
Dept. of Bioengineering
Univ. of Utah
50 So Campus Center Dr., Rm 2480 MEB
Salt Lake, UT 84112-9202

Re: 1 R21 RR017329-01

Dear Dr. Andrade:

Enclosed is a copy of the Summary Statement prepared by the Scientific Review Administrator of the Initial Review Group (IRG) that evaluated your application. It includes a numerical score and a narrative critique that reflect the IRG's evaluation of the scientific merit of your application.

This evaluation is the first step in a two-step evaluation process of peer review. The second step will be carried out by the National Advisory Research Resources Council at its meeting on May 16, 2002. The information in the Summary Statement, along with the recommendation from the Council, will be taken into consideration in making funding decisions. At this time, no conclusions should be drawn concerning the probability of funding. If the Council recommends an action that differs from the IRG evaluation, we will notify you specifically within 30 days after the Council meeting. Your application will remain in consideration for funding until the automatic withdrawal date note above.

Please note that the NIH has modified its policy regarding the receipt of amended applications. Amended applications beyond the second revision will no longer be accepted (NIH Guide, Vol. 25, No. 19, June 14, 1996).

If you have specific concerns about the review of your application, you are encouraged to contact Dr. Gregory Farber at (301) 435-0755 as soon as possible to discuss your options and obtain advice. If, after our discussion, you choose to write a response relevant to a serious concern, it must be received in this office no later than close of business Wednesday, May 1, 2002.

Sincerely,

Michael T. Marron, Ph.D.
Director, Biomedical Technology

Enclosure
cc: Vincent A. Bogdanski
Manager, Grants & Contracts

1&2

R21RR017329-01 Andrade, J.

RESUME AND SUMMARY OF DISCUSSION:
This application from the University of Utah proposes to develop microdevices capable of measuring up to 100 metabolites, with high sensitivity, in small volumes of biological fluids. It is intended to make these low cost devices using microfabrication and microfluidics, and to use bioluminescence as a detection method. This was considered a significant engineering proposal that combines microfluidics and microelectronics. The reviewers considered the novelty of combining existing techniques, the highly organized nature of design and methods, and the nature of the proposed studies on reagent stability to be the major strengths of this application. Concerns about the limitations of using polydimethylsiloxane (PDMS) as a manufacturing material, underestimation of the difficulty of microfabricating optics in a flexible polymer, and lack of enough attention to the nature of microfluidics reduced the enthusiasm for this proposal. The reviewers recommended support at the requested level.

DESCRIPTION (provided by applicant):
The investigators propose to develop microdevices for the specific chemical analysis of multiple metabolites in small sample volumes of biological fluids. The specificity and sensitivity is provided by specific reactions that couple analytes to bioluminescent-based enzyme reactions and produce light proportional to the analyte concentration. Bioluminescent analytical assays, in a miniaturized and stable format, can measure sub-microMolar concentrations in microliter sample volumes.

The goal of the first phase (R21) is to engineer microfluidic structures, develop enzyme packaging and stabilization techniques, and optimize optical detection systems in order to measure two model analyte solutions (galactose and lactate) using bioluminescent reactions.

The goal of the second phase (R33) is to implement other bioluminescent assays in the microfluidic detection system, develop specific diagnostic panels, utilize practical biofluid samples and enhance analytical accuracy and precision.

The proposed Micro-Analytical System (microAS) will be convenient to operate in point-of-care (POC) and home environments. It will likely evolve to measure up to 100 different metabolites in the submicroMolar to milliMolar range from one 1-100 microL biofluid sample, and include customized comprehensive diagnostic panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs.

CRITIQUE 1:
Significance: The development of the proposed micro-analytical system capable of measuring up to 100 metabolites in point-of-care and home environments with high sensitivity using very small sample volumes is an attractive goal since it can lead to better patient care at lower costs. Bioluminescence is a highly sensitive detection technique that can provide detection in the sub-micromolar concentration range. The power of the bioluminescent methods for routine chemical analysis has been hampered by the need for expensive luminometers. The proposed project aims to utilize microfabrication and microfluidics to create miniature, low-cost platforms that can provide detection below micromolar concentrations without luminometers. The integration of microfluidic structures with enzyme stabilization and optical detection will be a significant advance over current technology.

Approach: The investigators have planned out their research very well. They mention that their goal in the R21 phase is to realize the microfluidic structures, develop enzyme packaging and stabilization, and optimize optical detection. However, the engineering of microfluidic structures is not stated clearly in the Specific Aims; detection limit experiments, which are also important, are discussed instead. It is not

Gregory Farber
301-435-0778
farberg@ncrr.nih.gov

SUMMARY STATEMENT
(Privileged Communication)

Release Date: 03/29/2002

ANDRADE, JOSEPH D, PHD
UNIVERSITY OF UTAH
DEPT OF BIOENGINEERING
50 S CTRL CAMPUS DR RM 2480 MEB
SALT LAKE CITY, UT 84112-9202

Application Number: 1 R21 RR017329-01

Review Group: ZRR1 BT-1 (01)
National Center for Research Resources Special Emphasis Panel

Meeting Date: 02/26/2002
Council: MAY 2002
Requested Start: 05/01/2002

RFA/PA: PAR01-057
PCC: BTA230

Project Title: MULTI-ANALYTE MICRO-DEVICES FOR BIOMEDICAL APPLICATIONS

SRG Action: Priority Score: 195
Human Subjects: 10-No human subjects involved for competing applications
Animal Subjects: 10-No live vertebrate animals involved for competing appl.

Project Year	Direct Costs Requested	Estimated Total Cost
1	99,633	148,057
2	99,254	147,494
3	193,400	287,397
4	213,711	317,579
TOTAL	605,998	900,527

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

NOTE TO APPLICANT FOLLOWS SUMMARY STATEMENT

clear if the team has gained the expertise in soft lithography and related processing. If the answer is no, then the tasks to achieve Specific Aim 1 should incorporate fabrication process development, along with an appropriate milestone. The investigators should verify that the molded PDMS does not affect the enzyme properties. The effect of the silanizing agent on enzyme characteristics may not be negligible. The Preliminary Results are encouraging and justify the proposed project. However, the fabrication of quality photodetectors such as p-n, p-i-n, and avalanche photodiodes may not be feasible within an academic cleanroom. Consequently, the investigators should provide alternate strategies to overcome possible cleanroom processing problems. Commercial detection components that can be suitably configured by a technician or graduate student should be considered as a possible option. In the R33 phase, the project will be appropriately expanded to clinically relevant metabolites in blood and urine samples.

The inclusion of data analysis and visualization tools should ensure that results would be accurately presented. The approach to integrating the electrochemical and bioluminescent sensors on a single platform has not been detailed. However, this multifunctional sensor would be quite powerful and is accordingly encouraged. The success of the micro-analytical system for real world use will also be limited by system level performance of the packaged device – especially in terms of handling. It might be worthwhile for the investigators to incorporate tests that examine device operation after non-delicate handling, typical to the home or emergency room environments.

Milestones: The milestones are well written, quantifiable, and can be used to determine if the project should proceed to the R33 phase. The investigators have stated that results of the R21 phase will be presented at a number of international conferences as well as journals.

Innovation: The integration of microfluidic structures with enzyme stabilization and optical detection via bioluminescence will be a significant advance over current technology. Successful demonstration of the project milestones could revolutionize analysis of metabolites both within and outside the hospital. Consequently, it is not surprising that the investigators report interest in technology transfer from industry. Other applications for the device range beyond diagnosis and into environmental monitoring and industrial pollution assessment.

Investigator: The investigators are appropriately trained and qualified to complete the tasks for the projects. There is a question about expertise on soft lithography, but this lack of experience, if it does exist, can be overcome through collaboration.

Overall Evaluation: Bioluminescence is a highly sensitive detection technique that can provide detection in the sub-micromolar concentration range. The integration of microfluidic structures with enzyme stabilization and optical detection via bioluminescence will be a significant advance over current technology. The project is highly innovative and the tasks have been well planned out. The milestones are clearly written and quantifiable. The investigators have generated preliminary data that support further investigation.

CRITIQUE 2:
Significance: Rather than addressing a biomedical research problem, this study attempts to solve some very important engineering issues in point-of-care applications. If the aims are achieved there will be scientific knowledge gained in how to maintain reagent stability, how to optimally shape sensor cells/chambers, and how to avoid the use of expensive photomultiplier tubes.

Approach: Design and methods are extremely well developed. The applicant recognizes the potential problems and on several occasions suggests good back-up strategies. One area that is not sufficiently addressed is the driving force for the fluids to move from site to site. Capillary forces will not suffice.

With the proposed manufacturing method, few shapes of the optical cell will be enabled. Making arrays of photoconductors, p-n junction, p-i-n junctions, and avalanche photodiodes is complicated and expensive. It seems that the efforts required have been underestimated.

Innovation: The proposed work does not involve novel concepts, approaches or methods but constitutes a very good combination of interesting techniques that have been demonstrated separately. The combined aims are original and innovative. The project will not lead to a paradigm shift in research but could well develop new methodologies and technologies.

Investigator: This is a very qualified team. The team has the right background but they are perhaps trying to achieve more than what they are capable of.

Overall Evaluation: This is a very well written proposal that addresses an important set of engineering issues. The work that is proposed will provide some important references on reagent stability. This reviewer does not feel that PDMS is a good manufacturing material. It is only a good and fast prototyping material. The driving force for the fluidics part is not addressed properly. Some of the other weaknesses of the present application are lack of attention to real samples and sample preparation, and underestimation of the difficulty of microfabricating optics in a flexible polymer.

CRITIQUE 3:
The goal of the proposal is to engineer and fabricate microfluidic structures to be used with bioluminescent reactions. While the first phase goal (R21) concentrates on two model analyte solutions, the goal of the second phase (R33) is to implement other bioluminescent assays and be able to measure 100 different metabolites in the submicromolar, millimolar scale.

The proposal is well written. It combines a microfuidic expert (Andrade), with a microelectronic/photonics expert (Miller, Director of the Micromachining Laboratory), and pharmaceutics expert (Kern). This reviewer finds that one of the strengths of the proposal is to move from photo-multiplier tube (PMT) with low quantum efficiency to p-n, p-i-n as well as avalanche photodiodes with inherent high quantum efficiency. This comes as a result of the integration.

It is a nice and clean engineering proposal. The work is incremental and not novel as most of their work is described in International Society for Optical Engineering (SPIE) journals. However the outcome of this application will find a wide variety of applications in life science.

The milestones look reasonable, logical, and well conceived.

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW ADMINISTRATOR TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

MEETING ROSTER

National Center for Research Resources Special Emphasis Panel
NATIONAL CENTER FOR RESEARCH RESOURCES
ZRR1 BT-1 (01)
February 26, 2002 - February 27, 2002

CHAIRPERSON

JOHNSON, MARYL R., MD
ASSOCIATE PROFESSOR OF MEDICINE
MEDICAL DIRECTOR OF CARDIAC TRANSPLANT PROGRAM
NORTHWESTERN MEDICAL SCHOOL
CHICAGO, IL 60611

MEMBERS

BARBARA, PAUL F., PHD
PROFESSOR
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY
UNIVERSITY OF TEXAS
AUSTIN, TX 78712

BEEBE, DAVID J., PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF BIOMEDICAL ENGINEERING
UNIVERSITY OF WISCONSIN-MADISON
MADISON, WI 53706

BEEBE, THOMAS P. JR., PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY
UNIVERSITY OF DELAWARE
NEWARK, DE 19716

BHATIA, SANGEETA N., MD, PHD
DIRECTOR
MICROSCALE TISSUE ENGINEERING LABORATORY
UNIVERSITY OF CALIFORNIA-SAN DIEGO
LA JOLLA, CA 92093

COLTON, RICHARD J., PHD
HEAD, SURFACE CHEMISTRY BRANCH
CHEMISTRY DIVISION
NAVAL RESEARCH LABORATORY
WASHINGTON, DC 20375

DESCOUR, MICHAEL R., PHD
ASSOCIATE PROFESSOR
OPTICAL SCIENCES CENTER
UNIVERSITY OF ARIZONA
TUCSON, AZ 857210094

ECKSTEIN, EUGENE C., PHD
PROFESSOR
DEPARTMENT OF BIOMEDICAL ENGINEERING
UNIVERSITY OF MEMPHIS
MEMPHIS, TN 38152

GEORGIADIS, ROSINA M., PHD
PROFESSOR
DEPARTMENT OF CHEMISTRY
BOSTON UNIVERSITY
BOSTON, MA 02215

HARDIN, MICHAEL J, PHD
PROFESSOR
DEPARTMENT OF INFORMATION SYSYTEMS, STATIATICS,
AND MANAGEMENT SCIENCE
UNIVERSITY OF ALABAMA
300 ALSTON HALL
BOX 870226
TUSCALOOSA, AL 35487

HOFFMAN, ERIC P, PHD
JAMES CLARK PROFESSOR OF PEDIATRICS,
BIOCHEMISTRY & MOLECULAR BIOLOGY
GEORGE WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, AND
DIRECTOR, RESEARCH CENTER FOR GENETIC MEDICINE
CHILDREN'S NATIONAL MEDICAL CENTER
WASHINGTON, DC 20010

LEWIN, PETER A, PHD
PROFESSOR AND DIRECTOR
DREXEL UNIVERSITY
SCHOOL OF BIOMEDICAL ENGINEERING, SCIENCE AND HEALTH SYSTEMS AND
THE DEPARTMENT OF ELECTRICAL AND COMPUTER ENGINEERING
PHILADELPHIA, PA 19104

LI, SHI-JIANG W., PHD
ASSOCIATE PROFESSOR
BIOPHYSICS RESEARCH INSTITUTE
MEDICAL COLLEGE OF WISCONSIN
MILWAUKEE, WI 53226

LIANG, JEROME Z, PHD
PROFESSOR
DEPARTMENT OF RADIOLOGY
STATE UNIVERSITY OF NEW YORK
STONY BROOK, NY 117948460

MADOU, MARC J., PHD
VICE PRESIDENT
OF ADVANCED TECHNOLOGY
NANOGEN, INC.
SAN DIEGO, CA 92121

MATHIOWITZ, EDITH , PHD
PROFESSOR
DEPARTMENT OF MOLECULAR PHARMACOLOGY AND BIOTECHNOLOGY
BROWN UNIVERSITY
PROVIDENCE, RI 02912

NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address: http://grants.nih.gov/grants/policy/amendedapps.htm

NIH announced implementation of Modular Research Grants in the December 18, 1998 issue of the NIH Guide to Grants and Contracts. The main feature of this concept is that grant applications (R01, R03, R21, R15) will request direct costs in $25,000 modules, without budget detail for individual categories. Further information can be obtained from the Modular Grants Web site at http://grants.nih.gov/grants/funding/modular/modular.htm

MOTAMEDI, MASSOUD , PHD
DIRECTOR
CENTER FOR BIOMEDICAL ENGINEERING
UNIVERSITY OF TEXAS
MEDICAL BRANCH AT GALVESTON
GALVESTON, TX 77555

MOUROU, GERALD , PHD
PROFESSOR
DEPARTMENT OF ELECTRICAL ENGINEERING
AND COMPUTER SCIENCE
UNIVERSITY OF MICHIGAN
ANN ARBOR, MI 48109

NELSON, SARAH J., PHD
PROFESSOR
MAGNETIC RESONANCE SCIENCE CENTER
DEPARTMENT OF RADIOLOGY
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
SAN FRANCISCO, CA 941431290

NISHIKURA, KAZUKO , PHD
PROFESSOR
DEPARTMENT OF MOLECULAR GENETICS
THE WISTAR INSTITUTE
PHILADELPHIA, PA 19104

OBER, CHRISTOPHER K., PHD
PROFESSOR
DEPARTMENT OF MATERIALS
SCIENCE AND ENGINEERING
CORNELL UNIVERSITY
ITHACA, NY 14853

OHNO-MACHADO, LUCILA , PHD
ASSISTANT PROFESSOR OF RADIOLOGY
DECISION SYSTEMS GROUP
BRIGHAM AND WOMEN'S HOSPITAL
HARVARD MEDICAL SCHOOL
BOSTON, MA 02115

ROY, SHUVO , PHD
CO-DIRECTOR, BIOMEMS LABORATORY
DEPARTMENT OF BIOMEDICAL ENGINEERING(ND20)
LERNER RESEARCH INSTITUTE
CLEVELAND, OH 44195

TARLOV, MICHAEL J., PHD
LEADER, PROCESS SENSING GROUP
NATIONAL INSTITUTE OF STANDARDS
AND TECHNOLOGY
GAITHERSBURG, MD 20899

WEBSTER, JOHN G., PHD
PROFESSOR
DEPARTMENT OF BIOMEDICAL ENGINEERING
UNIVERSITY OF WISCONSIN
MADISON, WI 53706

MAIL REVIEWER(S)

DESALLES, ANTONIO A. F., MD, PHD
PROFESSOR
DIVISION OF NEUROSURGERY
UCLA MEDICAL CENTER
LOS ANGELES, CA 90095

LOVETT, MICHAEL A, PHD
PROFESSOR OF GENETICS
DEPARTMENT OF GENETICS
WASHINGTON UNIVERSITY
SCHOOL OF MEDICINE
ST. LOUIS, MO 63110

RUBINSKY, BORIS , PHD
PROFESSOR
DEPARTMENT OF MECHANICAL ENGINEERING
UNIVERSITY OF CALIFORNIA
BERKELEY, CA 94720

SEVILLA, MICHAEL D., PHD
PROFESSOR AND CHAIR
CHEMISTRY DEPARTMENT
OAKLAND UNIVERSITY
ROCHESTER, MI 48309

VAN ZIJL, PETER C, PHD
PROFESSOR
DEPARTMENT OF RADIOLOGY
JOHNS HOPKINS SCHOOL OF MEDICINE
BALTIMORE, MD 21205

SCIENTIFIC REVIEW ADMINISTRATOR

VISWANATHAN, MOHAN , PHD
SCIENTIFIC REVIEW ADMINISTRATOR
NATIONAL CENTER FOR RESEARCH RESOURCES
NATIONAL INSTITUTES OF HEALTH
6705 ROCKLEDGE DRIVE, MSC 7965
BETHESDA, MD 20892

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

Principal Investigator/Program Director (Last, First, Middle): Andrade, Joseph D.

PROGRESS REPORT SUMMARY

GRANT NUMBER: 5R33RR017329-04

PERIOD COVERED BY THIS REPORT

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	FROM	THROUGH
Joseph D. Andrade, Ph.D.	8/1/04	7/31/05

APPLICANT ORGANIZATION
University of Utah: Department of Bioengineering

TITLE OF PROJECT (Repeat title shown in Item 1 on first page)
Multi-Analyte Micro-Devices for Biomedical Applications

A. Human Subjects (Complete Item 6 on the Face Page)
Involvement of Human Subjects ☒ No Change Since Previous Submission ☐ Change

B. Vertebrate Animals (Complete Item 7 on the Face Page)
Use of Vertebrate Animals ☒ No Change Since Previous Submission ☐ Change

SEE PHS 2590 INSTRUCTIONS.
WOMEN AND MINORITY INCLUSION: See PHS 398 Instructions. Use Inclusion Enrollment Report Format Page and, if necessary, Targeted/Planned Enrollment Format Page.

A. Specific Aims

Specific aims for the R33 Phase have not been modified from the original application:

- *Specific Aim 1* – ChemChip Analyte/Panel Development – develop assays for additional important metabolites, nutrients, and therapeutic drugs, including a kidney/hemodialysis panel;
- *Specific Aim 2* – Blood and Urine Samples – deal with urine calibration issues and blood analysis separation, and calibration;
- *Specific Aim 3* – Quality Assurance – develop means for on-board chip calibration, enhance the stability of the chips for long term storage, and deal with detection range features;
- *Specific Aim 4* – Data Analysis and Multi-Parameter Visualization – what we commonly call InfoWare – develop multi-parameter visualization tools; particularly multi-axes radar plots to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

Citations are listed in Section E on page 9.

B. Studies and Results

ChemChip Fabrication Update: [9]

ChemChip prototypes were made by cutting holes in adhesive films and applying them to glass cover slides [4]. Exploration of this prototyping method resulted in layered microfluidic structures (down to 20 μm) that can be laminated in less than 30 minutes without any photolithographic processes or chemicals [3, 6].

Detector Update: [9]

Previously, we used a backed-thinned cooled CCD to measure signals [4]. We now have an inexpensive detector based on commercial photodiode arrays. The initial detector used transimpedance amplifiers but had significant noise levels. The current circuit combines the photodiode arrays with integrating op-amps that have built in analog to digital converters (Texas Instruments DDC112). Close proximity of the photodiodes and digital converters reduced noise levels enough to detect photocurrents as low as 50 pA. Initial tests were performed with radioluminescent standards. We are also testing the bioluminescent reagents.

Specific Aim 1:

Basic Metabolism Panel [8, 11]

Our basic metabolism panel includes glucose, lactate, creatine, creatinine, pyruvate, and glutamine via bioluminescence-based reactions already established. We have improved detection range of the lactate assay and reduced the error from 22% to 12%. We are working to do the same for the other assays.

Kidney & Hemodialysis Panel: [7, 8]

We developed glucose and phosphate chemiluminescence-based assays and performed kinetic modeling [2, 18] to optimize reagent concentrations for the bioluminescence-based creatinine, creatine, and urea assays. Standard curves and errors were determined for each assay.

Antiepileptic Drug Application: [5, 10]

We have developed a one-step bioluminescence-based homogeneous immunoassay platform for measuring antiepileptic drugs (AEDs) by modifying a Cloned Enzyme Donor Immunoassay (CEDIA) [1-2, 14-15]. The platform was developed by combining the β-galactosidase of the CEDIA with the Beta-Glo Assay System (Promega Corp.) [16-17]. The Beta-Glo assay utilizes a luciferin-galactoside substrate (6-O-β-galactopyranosyl-luciferin). The one-step CEDIA with Beta-Glo assay system was used to measure three traditional AEDs – carbamazepine (CBZ), phenytoin (PHT), and valproic acid (VPA). Simulations and experiments of the one-step assay resulted in a larger detection range, within a shorter time frame, and with less reagent compared to the commercial Microgenics Corp. two-step colorimetric CEDIA.

Steroid Hormone Applications: [12]

We tested the homogeneous DiscoveRx HitHunter cortisol assay and measured a detection range of 10^{-10}-10^{-6} M.

Specific Aim 2: [10]

Serum and plasma samples spiked with CBZ, PHT, and VPA were measured by the bioluminescence-based immunoassay. A 90% reduction in the activity level was observed due to protein binding and interference from endogenous serum/plasma compounds. Albumin binding characteristics of the three AEDs measured with this immunoassay platform will be simulated and tested to determine the extent of protein-drug binding as a function of protein and drug concentrations. This will help to distinguish free (unbound) from total (free plus bound) drug concentrations. Other drugs known to compete with the AEDs for albumin binding sites will also be tested.

Specific Aim 3: [9, 11, 13]

We are developing on-board calibration methods in order to account for effects from sample interactions and storage conditions. Calibration wells are created by freezing the bioluminescent reagents in a ChemChip well. An excipient solution spiked with a known amount of analyte is then frozen on top of the reagent and all the layers are freeze dried. When the sample dissolutes reagents in the calibration well, it produces a luminescent signal proportional to the unknown analyte concentration plus the known spiked amount. At least one calibration well is required for each parameter of the model calibration curve. The unknown concentration and the model parameters are calculated by linear regression. This method was used to test on-board calibration of the galactose assay. The result showed a -18% measurement error from a 180 μM sample. Accuracy can be improved by including more calibration wells or improving the model.

C. Significance

The rapid prototyping method discovered during ChemChip development is an inexpensive method that other laboratories can use to design and test their own microfluidic based biosensors [3-4, 6, 9].

The one-step bioluminescence-based immunoassay system provides a highly sensitive, simple, rapid and inexpensive platform, making it suitable for point-of-care monitoring of drugs and steroids [1, 5, 10].

On-board calibration standards for assays lyophilized on ChemChips will help ensure measurement accuracy, independent of batch fabrication and sample matrix effects, while maintaining ease of use at the point of care [9, 11, 13].

D. Plans

Specific Aim 1:

Multiple analytes will be tested on single ChemChips. The steroid assays will be converted to a single step bioluminescent assay in order to simplify the process and reduce measurement time. Kinetic modeling will be used to optimize reagent ratios in order to extend the detection range for hyper and hypo steroid levels.

Specific Aim 2:

Blood and urine tests will be performed on the basic metabolism panel and kidney & hemodialysis panel to test interference effects. Similar tests will be done for the Immunoassay based ChemChips.

Specific Aim 3:

Multiple assays for the steroid, drugs, and metabolite analytical panels will be tested together on ChemChips in their lyophilized form. Long term storage studies will also be performed.

Specific Aim 4:

We will use radar plots to visualize relationships between analytes on a panel by normalizing ranges of analyte measurements to a common scale. Normal, below normal, and above normal indicators will be included on each scale.

E. Publications & References

Starred (*) references are included with the report.

Grant Generated Publications

Journal Article:

1. *Yang X, Janatova J, Andrade J.D., "Homogeneous enzyme immunoassay modified for application to luminescence-based biosensors," *Analytical Biochemistry* 2005, 336(1):102-7.
2. Jeon SI, Yang X, Andrade JD. Modeling of homogeneous cloned enzyme donor immunoassay. *Analytical Biochemistry* 2004; 333: 136-147. (Previously reported and submitted).

Journal Article (Submitted):

3. *Bartholomeusz, D., Boutté, R., and Andrade, D., "Xurography: Rapid Prototyping of Micro-Structures Using a Cutting Plotter," IEEE Journal of Microelectromechanical Systems (JMEMS), (Review received April 2005, in revision).

Conferences:

4. *Bartholomeusz D., and Andrade, J., "Photodetector Calibration Method For Reporting Bioluminescence Measurements In Standardized Units," *Proceedings of the XIIIth International Symposium on Bioluminescence and Chemiluminescence: Progress and Perspectives*, August 2-6, 2004, Yokohama, Japan, *Luminescence*, World Scientific Publishing, 233-236
5. *Yang X, Janatova J, Andrade J.D., "Development of therapeutic drug monitoring using disposable ChemChip devices: potential application to drug delivery systems" (Poster). *Twelfth International Symposium on Recent Advances in Drug Delivery Systems 2005*, Salt Lake City, UT, USA.

Provisional Patent Application:

6. *University of Utah Invention Disclosure U-3760: "Rapid Prototyping of Micro-Structures Using a Cutting Plotter: Materials and Methods." Patent Application # 60/669570. This provisional is currently in the process of being filed and will be reported according to the invention reporting compliance requirements.

Published Thesis:

7. R Davies, PhD., Dec., 2004: Luminescent Assays for the Assessment of Metabolism and Renal Function: Towards a Multi-Analyte Biosensor.

Theses in Preparation:

8. P. Mohan, MS., Fall, 2005: Toward a renal chip for assessment of kidney function
9. D. Bartholomeusz, PhD., Spring, 2006: Fabrication of a Bioluminescence-Based Multi-Analyte Biosensor
10. X.Y. Yang, PhD., Summer, 2006: Towards the Development of an ImmunChip for Anti-Epileptic Drugs.
11. Y. Al-Sheikh, PhD., Fall, 2006: Calibration, Data, Visualization, and Interpretation of a Multi-Analyte, Luminescence-Based ChemChip for Assessing and Monitoring Metabolic Diseases
12. J. Jensen, PhD., Winter, 2006: Toward Steroid Hormone Applications of Personal Multi-analyte Biosensors.
13. M. Yang, MS., Fall, 2006: Process and Fabrication Issues in the Preparation and Production of ChemChips

Other References

14. Henderson DR, Friedman SB, Harris JD, Manning WB, Zoccoli MA., "CEDIA, A New Homogeneous Immunoassay System," *Clin Chem* 1986; 32: 1637-1641.
15. Wiegand RF, Klette KL, Stout PR, Gehlhausen JM. Comparison of EMIT II, "CEDIA, and DPC RIA Assays For The Detection Of Lysergic Acid Diethylamide In Forensic Urine Samples.," *J Anal Toxicol* 2002; 26: 519:523
16. Geiger R, Schneider E, Wallenfels K, Miska W, "A New Ultrasensitive Bioluminogenic Enzyme Substrate for Beta-Galactosidase," *Biol Chem Hoppe Seyler* 1992; 373:1187-1191.
17. Hannah R, Stroke I, Betz N, "Beta-Glo® Assay System: A Luminescent B-Galactosidase Assay For Multiple Cell Types and Media," *Cell Notes* 2003; 6: 16-18.
18. Mendes, P. (1993) GEPASI: A software package for modelling the dynamics, steady states and control of biochemical and other systems. *Comput. Applic. Biosci.* 9, 563-571

F. Project Generated Resources

There were no project generated resources during this reporting period.

KEY PERSONNEL REPORT

Place this form at the end of the signed original copy of the application. Do not duplicate.

GRANT NUMBER: 5R33RR017329-04

All Key Personnel for the Current Budget Period (do not include Other Significant Contributors)

Name	Degree(s)	SSN (last 4digits)	Role on Project (e.g. PI, Res. Assoc.)	Date of Birth (MM/DD/YY)	Annual % Effort
Joseph D. Andrade	BS, PhD	0772	PI	07/13/41	25.0
Steven Kern	BS,MS, PhD	9840	Co-investigator	07/24/61	15.0

Principal Investigator/Program Director (Last, first, middle): Andrade, Joseph D.

PROGRESS REPORT SUMMARY

GRANT NUMBER
1 R21 RR 17329-02

PERIOD COVERED BY THIS REPORT

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	FROM	THROUGH
Joseph D. Andrade, Ph.D.	8/1/03	7/31/04

APPLICANT ORGANIZATION
University of Utah: Department of Bioengineering

TITLE OF PROJECT (Repeat title shown in Item 1 on first page)
Multi-Analyte Micro-Devices for Biomedical Applications

A. Human Subjects (Complete Item 6 on the Face Page)
Involvement of Human Subjects ☒ No Change Since Previous Submission ☐ Change

B. Vertebrate Animals (Complete Item 7 on the Face Page)
Use of Vertebrate Animals ☒ No Change Since Previous Submission ☐ Change

SEE PHS 2590 INSTRUCTIONS.
WOMEN AND MINORITY INCLUSION: See PHS 398 Instructions. Use Inclusion Enrollment Report Format Page and Targeted/Planned Enrollment Format Page.

1) There is no change in the other support of key personnel since the last reporting period.
2) There will not be, in the next budget period, a significant change in the level of effort for key personnel from what was approved for this project. There are no additional Key Personnel involved.
3) It is not anticipated that an estimated unobligated balance (including prior year carryover) will be greater than 25 percent of the current year's total budget (indeed, it will be about 0%).

MILESTONES for R21 (from Notice of Grant Award):

1) A microdevice with appropriate reservoirs will be fabricated using PDMS. This device will allow the concentration of ATP, NADH, galactose, and lactate to be determined using bioluminescent based chemical assays. A CCD, or another detector array, will be used as the detector for these assays. For ATP, the device must be able to detect 10 picomoles with a maximum error of 1 picomole. For NADH, the device must be able to detect 200 picomoles with a maximum error of 20 picomoles. For galactose, the device must be able to detect 20 picomoles in a 1 microliter volume with a maximum error of 2 picomoles. The device must be able to determine galactose concentrations in the range from 20 to 90 micromolar. For lactate, the device must be able to detect 500 picomoles in a 1 microliter volume with a maximum error of 50 picomoles. The device must be able to determine lactate concentrations in the range from 0.5 to 2.5 millimolar;

2) Using the microdevice fabricated in milestone 1, methods will be developed to lyophilize the assay mixture in the microdevice. At the conclusion of this milestone, the following detection limits should be achievable using a lyophilized assay mixture. For ATP, the device must be able to detect 100 picomoles with a maximum error of 20 picomoles. For NADH, the device must be able to detect 2 nanomoles with a maximum error of 400 picomoles. For galactose, the device must be able to detect 200 picomoles in a 1 microliter volume with a maximum error of 40 picomoles. The device must be able to determine galactose concentrations in the range from 200 to 900 micromolar. For lactate, the device must be able to detect 5 nanomoles in a 1 microliter volume with a maximum error of 1 nanomole. The device must be able to determine lactate concentrations in the range from 5 to 25 millimolar. The sample fluid for these assays must flow from an input port and fill each well without any technical difficulty or complicated pumps. The sample fluid must fill greater than 95% of the wells without bubbles. A CCD, or another detector array, will be used as the detector for these assays; and

3) Build a detector array or adapt a commercially available detector array and coupling mechanism for the microdevice fabricated in milestone one. This array will be used to measure the concentrations of ATP, NADH, galactose, and lactate using lyophilized assay mixtures. For ATP, the device must be able to detect 10 picomoles with a maximum error of 2 picomoles. For NADH, the device must be able to detect 200 picomoles with a maximum error of 40 picomoles. For galactose, the device must be able to detect 20 picomoles in a 1 microliter volume with a maximum error of 4 picomoles. The device must be able to determine galactose concentrations in the range from 20 to 90 micromolar. For lactate, the device must be able to detect 500 picomoles in a 1 microliter volume with a maximum error of 1 picomole. The device must be able to determine lactate concentrations in the range from 0.5 to 2.5 millimolar.

MILESTONE RESULTS

1) Microdevices with Wet Reagents:

The ChemChip fabrication was simplified since last year's progress report for cost effectiveness and prototyping flexibility. 15mm squares with a 5x5 array of 1mm diameter holes spaced 2 mm apart, were cut out of 0.180 mm thick adhesive backed vinyl film with a knife plotter used for conventional vinyl sign making. The ChemChip patterns were then sealed to 15mm square glass cover slips after manually removing the cut holes. The glass cover slips became the clear bottom for the 140 nL wells (Figure 1).

The chips were loaded, both manually and via a computer controlled syringe pump/solenoid auto dispensing system (Figure 2), with the needed reagent cocktails. A miniature solenoid valve with a 0.002" nozzle (the Lee Company) was used to dispense the reagents (10 ms pulses, pressurized at 8 PSI). The resultant drops were calibrated at 360±10nL. A computer controlled XYZ stage positioned a tray of 25 ChemChips and dispensed reagent drops into individual wells (Figure 2).

The detection limits for wet ATP and NADH were already met in last year's Progress Report; even lower detection limits were achieved recently using a commercial CCD imaging detector.

2) Microdevices with Lyophilized Reagents:

The detection limits for dry ATP and NADH were already met in last year's Progress Report. We have optimized the lyophilization protocols and applied them to the fabrication of multi-channel ChemChips appropriate to Milestone 2.

The reagents were dispensed as noted above onto a tray of ChemChips cooled to less than –60 degrees C using dry ice (Figure 2 left shows the tray of chips on the cold plate)—this allows the reagent droplets to freeze within seconds of dispensing. This process prevented evaporation and maintained reagent stability prior to lyophilization. Since drops were larger than the volume of the wells, a convex meniscus formed above each well.

Lyophilization was performed in two stages in a VirTis Genesis 12 pilot plant lyophilizer. The chips were placed in the sample chamber of the lyophilizer, which had been previously cooled to at least –50° C. Primary lyophilization began when the sample chamber was connected to the condenser chamber cooled to at least -70° C and the system pressure was below 100 milliTorr. Primary lyophilization was performed for 48-72 hours. After the temperature of the sample chamber is changed to 25 ° C, secondary lyophilization was performed for 12-24 hours.

The lyophilized reagents rapidly rehydrate when sample fluids are added to the top of the array. In other studies dealing with analytes in hydrogel contact lenses, we realized that the ChemChip can be easily filled from the top – vertical delivery. We adopted this approach to sample delivery rather than a directional microfluidic approach for simplicity and reliability—this led to the Chip design already discussed (Figure 1). We thus deliver samples to the dry ChemChips using 14 mm diameter circles of Whatman filters; the filters were clamped onto the center of the ChemChips. 25uL samples were dispensed on the center on the membrane. The membranes hydrate uniformly; less that 1 μL of the 25μL sample is available for each of the 25 wells. Given a 2mm well spacing and a 0.2mm thick membrane, up to 510 nL is delivered to any given well. The sample wicks along the membrane and in to each well whereupon the reagents rehydrate and the bioluminescence reactions begin. An optimum process results in a convex lyophilized reagent surface, which facilitates drawing the samples into each of the wells from the membranes (this process will be more fully optimized in the next several months). The

porous and hydrophilic structure of the lyophilized reagents and filter allowed the sample to uniformly fill the wells without bubble formation.

A commercially available Andor DV-434 CCD was used to take a kinetic series of 30-second exposures of the bioluminescence activity for each assay. Typical CCD Images can be seen in Figure 3.

The analytical results are summarized in Table I and Figures 4-6. The Milestone 2 detection limits were exceeded by at least a factor of 20 for all 4 analytes. Although the average error reported for galactose and lactate exceed 20% in this data, had we not pushed the detection limit (we got carried away), we would have come in well under 20%. A major component of the error is sample volume delivery, which we are now addressing using more controlled means for vertical sample delivery

3) Microdevice Detector Arrays:

This milestone was easily met using the Andor DV-434 CCD (Table 1). We have preliminarily evaluated two much less expensive detector arrays: Hamamatsu S8550 and S7585 photodiode arrays. The assays produced an estimated 10 nanoWatts/steradian/cm^2. Such a signal produces a current signal of about 50 pA (about 50 times greater than the dark current) on the Hamamatsu S8593 and S8550 photodiode arrays (given a collection angle of 1 steradian, an area of 5.3mm^2, and a photosensitivity of 0.3 A/W). Given the results reported above, these new arrays will be adequate and will greatly simplify the detection instrument – they are now under extensive evaluation and are the focus of the work in June and July, 2004).

PHASE II (R33) RESEARCH PLANS

R33 Abstract:

We propose to continue the development of microdevices for the specific chemical analysis of multiple metabolites in small sample volumes of biological fluids. The bioluminescent-based enzyme reactions developed in the R21 Phase will be augmented by bioluminescent immunoassays to greatly extend the clinical applicability of the ChemChip system. The Phase 1 (R21) Milestones were successfully met.

The goal of the second phase (R33) is to evaluate and utilize much less expensive array detectors, implement other bioluminescent assays including immunoassays, develop specific diagnostic panels, utilize practical biofluid samples, enhance analytical accuracy and precision, and develop effective means of presentation of the multi-analyte data to the caregiver and patient.

Specific Aim 1—Analyte/Panel Development—will provide assays for additional important metabolites, nutrients, and therapeutic drugs, including a kidney/hemodialysis panel;

Specific Aim 2 – Blood and Urine Samples – will deal with urine calibration issues, blood separation and calibration, and tear sample analysis and calibration;

Specific Aim 3 – Quality Assurance – will develop means for on board chip calibration, enhance the stability of the chips for long term storage, and deal with range features;

Specific Aim 4 – Data Analysis and Multi-Parameter Visualization – what we commonly call InfoWare –

will develop multi-parameter visualization tools; particularly multi-axes radar plots to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

Figures and Tables

Table 1: Detection Limits and Ranges Achieved for ATP, NADH, Galactose, and Lactate in Lyophilized Bioluminescence-Based Assays

Analyte	Required Detection Range	Detectable Range Tested	Sample Volume (maximum of 1 µL)	Experimental Detection Limit (lyophilized form)	Average Error	Detection Limit required for Milestone 2 (lyophilized assays) (error <± 20%)	Detection Limit required for Milestone 3 (lyophilized assays) (error <± 20%)
ATP	Down to 0.001 mM	Linear from 0.001 to 0.1 mM	0.51 µL	0.51 picomoles	19%	100 picomoles	10 picomoles
NADH	Down to 0.1 mM	Linear from 0.01 to 1.0 mM	0.51 µL	5.1 picomoles	21%	2,000 picomoles	200 picomoles
Galactose	0.020 to 0.090 mM	Linear from 0.1 to 1.0M. Logarithmic from 0.01 to 0.1 mM.	0.51 µL	5.1 picomoles	26%	200 picomoles	20 picomoles
Lactate	0.5 to 2.5 mM	Linear from 0.1 to 10 mM	0.51 µL	51 picomoles	22%	5,000 picomoles	500 picomoles

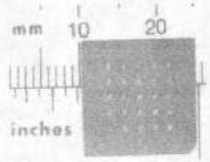


Figure 1: Empty ChemChip

A 5x5 array of 1mm diameter holes were cut from adhesive backed vinyl and sealed to a 15mm square glass cover slip. The notch on the bottom right corner is for indexing the wells.

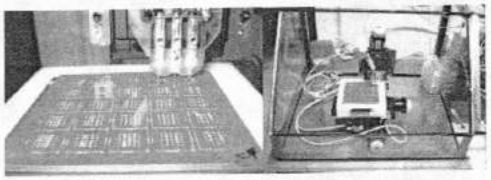


Figure 2: Reagent Deposition System

A tray holding 25 ChemChips (left) is positioned under a set of miniature solenoid valves with 0.002" nozzles by a computer controlled XYZ stage (right). The tray sits on a copper box filled with dry ice so the assays freeze almost instantly. If the reagents are allowed to thaw or evaporate, the reagent's convex surface collapses resulting less effect sample delivery.

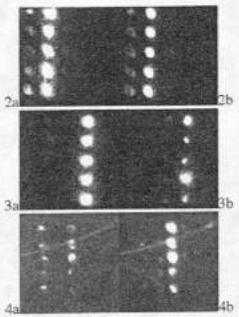


Figure 3: Chemchip Design and CCD Measurements of Bioluminescence Activity

Column	Analyte
1,N	NADH
2,A	ATP
3,L	Lactate
4,G	Galactose
5,B	Blank

1) (right drawing) Bioluminescence assay were dispensed in separate columns in order to get replicate data (5 rows per column).

(left pictures)

2a) NADH and ATP at 1 and 0.1 mM, respectively.

2b) NADH and ATP at 0.01 and 0.001 mM, respectively. This is dimmer than 2a due to lower concentration of analytes.

3a) Galactose assay (1mM sample) at first 30-sec exposure.

3b) Galactose assay (1mM sample) at 6th 30-sec exposure. Being a competition assay, the luminescence decreases with time.

4a) Lactate assay (10mM sample) at first 30-sec exposure. (The streaks of light across the images are due to a cracked cover slip).

4b) Lactate assay (10mM sample) at 6th 30-sec exposure. Being a production assay, the luminescence increases with time.

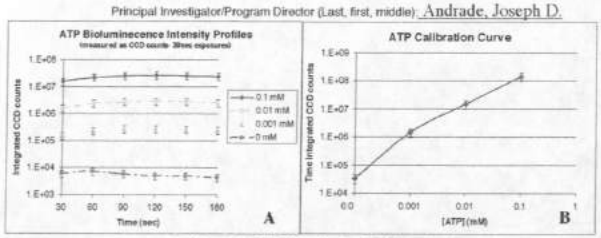


Figure 4: ATP Kinetics And Calibration Curve

25μL samples containing different concentrations of NADH, ATP, Lactate, and Galactose were delivered to each ChemChip by pipetting the sample onto a 14mm diameter filter membrane clamped onto the ChemChip. Six 30-second exposures were then taken using an Andor DV-434 CCD cooled to -50°C. The CCD counts over the area for each well were then integrated for each time frame. The integrated CCD counts were then averaged across all 5 rows for each column of analyte. An average integration was also taken across multiple chips that were tested at each sample concentration.

A) The intensity time profile for ATP bioluminescence, averaged across multiple chips (n=3) and 5 rows per chip, at 4 concentrations (including a control).

B) This ATP concentration calibration curve calculated by integrating the CCD counts for all six exposures, at each concentration. Given a sample of less than 510 nL, we were able to collect signals that were almost two orders of magnitude greater than the noise level, resulting in a detection limit of 0.51 picomoles. The calibration curve is linear for measurements covering three orders of magnitude.

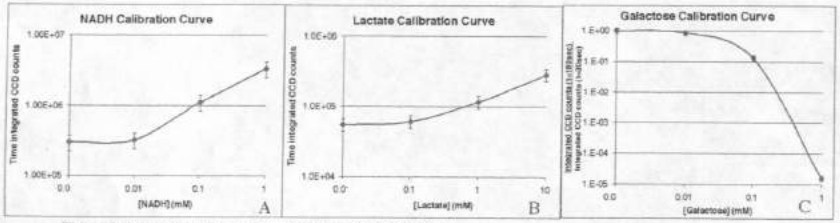


Figure 5: NADH, Lactate, and Galactose Calibration Curves

A) The NADH calibration curve shows a linear response from 0.01 to 1 mM. A 0.01 mM NADH sample (510nL) resulted in a detection limit of 6.4 picomoles. (0.01mMx510nL=5.1picomoles).

B) The Lactate calibration curve shows a linear response from 0.1 to 10nM with a detection limit of 6.4 picomoles

C) Because the Galactose assay was a competition assay, the calibration curve is based on the integrated CCD counts at t=180sec, divided by the integrated CCD counts at t=30sec (the brightest exposure period). This graph shows a linear response between 0.1 and 1 mM. As the Galactose concentration approaches 0.01mM, response is less linear. This non-linear region can be seen in Figure 6, which is the same data in Figure 5C without plotting the Y-axis on a log-scale.

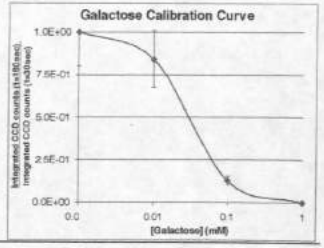


Figure 6 (right): Galactose Calibration Curve (Y-Axis not on Log Scale)
With the Y-axis plotted on a linear scale, we can better see the limits of detection and errors for the Galactose assay as the sample concentration approaches 0.01mM. We estimate a detection limit of 5.1 picomoles (0.01mMx510nL = 5.1 picomoles).

The **Specific Aims** for the R33 Phase II have not been modified from the original application. The details for each of the Specific Aims were presented in the original proposal.

Significance:
The ChemChip system will provide comprehensive diagnostic and monitoring panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs. The development of panels appropriate to specific clinical research areas, such as the kidney/hemodialysis panel, will greatly facilitate such research, due to the ease of use, low cost, and multi-parametric data generation provided by ChemChip devices. When more fully developed and available, ChemChips can be considered a research resource for a wide range of clinical and biochemical studies. A specific example is the thesis now underway by Jensen (see title below), potentially applying ChemChips to endocrine disorders.

Publications (*=copy is attached)
Journal Article:

R. Davies, D. Bartholomeusz, J. Andrade, "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" IEEE Engineering in Medicine and Biology Magazine, Volume 22, Issue 5, Jan/Feb (2003), pg 33-43.(this paper was cited and submitted with last year's Progress Report)

Journal Article (accepted and in press):
*Sang IL Jeon, Xiaoyun Yang and Joseph D. Andrade, "Modeling of Homogeneous Immunoassay (CEDIA)", Analytical *Biochemistry, accepted May, 2004*.Journal Article (submitted):

*Xiaoyun Yang, Jarmila Janatova, and Joseph D Andrade, "Single Step CEDIA using Spectrophotometric, Chemiluminescent, and Bioluminescent Substrates," submitted to J Immunologic Methods, May, 2004.Conference Proceedings:

Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units, D. Bartholomeusz and JD Andrade, in PE Stanley and LJ Kricka, eds., Bioluminescence and Chemiluminescence, World Scientific, (2002), pp. 189-192 (this paper was presented with the last Progress Report)

*Lactate Assay based on Bacterial Bioluminescence: Enhancement, Dry Reagent Development, and Miniaturization, RH Davies, JW Corry, JD Andrade, Ibid., pp. 441-444. (although this paper and the next did not cite support of this grant, they should have, as the work was largely supported by this grant—we apologize for the oversight)

*Enzyme Kinetics Model of the Bacterial Luciferase Reactions for Biosensor Applications, Y Feng, RH Davies, JD Andrade, Ibid., p. 88.

Abstracts:

*Bartholomeusz DA, Davies RH, Al-Sheikh YT, and Andrade JD, "Development of a Multi-Analyte ChemChip for Metabolic Assessment and Monitoring," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 231.

*Abernathy, JB, Davies, R, and Andrade J, ""Bioluminescence-Based Glucose Assay for a Multi-Analyte Biosensor," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 227.

Davies, RH Andrade, JD, "Diabetes-Related Luminescent Assays for Multi-Analyte Measurement" in *Diabetes Technology & Therapeutics*, 2003, **6**(2): p. 237

Abstracts Submitted:

will develop multi-parameter visualization tools; particularly multi-axes radar plots to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

The **Specific Aims** for the R33 Phase II have not been modified from the original application. The details for each of the Specific Aims were presented in the original proposal.

Significance:
The ChemChip system will provide comprehensive diagnostic and monitoring panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs. The development of panels appropriate to specific clinical research areas, such as the kidney/hemodialysis panel, will greatly facilitate such research, due to the ease of use, low cost, and multi-parametric data generation provided by ChemChip devices. When more fully developed and available, ChemChips can be considered a research resource for a wide range of clinical and biochemical studies. A specific example is the thesis now underway by Jensen (see title below), potentially applying ChemChips to endocrine disorders.

Publications
Journal Article:

R. Davies, D. Bartholomeusz, J. Andrade, "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" IEEE Engineering in Medicine and Biology Magazine, Volume 22, Issue 5, Jan/Feb (2003), pg 33-43.(this paper was cited and submitted with last year's Progress Report)

Journal Article (accepted and in press):
*Sang IL Jeon, Xiaoyun Yang and Joseph D. Andrade, "Modeling of Homogeneous Immunoassay (CEDIA)", Analytical *Biochemistry, accepted May, 2004.*Journal Article (submitted):

*Xiaoyun Yang, Jarmila Janatova, and Joseph D Andrade, "Single Step CEDIA using Spectrophotometric, Chemiluminescent, and Bioluminescent Substrates," submitted to J Immunologic Methods, May, 2004.Conference Proceedings:

Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units, D. Bartholomeusz and JD Andrade, in PE Stanley and LJ Kricka, eds., Bioluminescence and Chemiluminescence, World Scientific, (2002), pp. 189-192 (this paper was presented with the last Progress Report)

*Lactate Assay based on Bacterial Bioluminescence: Enhancement, Dry Reagent Development, and Miniaturization, RH Davies, JW Corry, JD Andrade, Ibid., pp. 441-444. (although this paper and the next did not cite support of this grant, they should have, as the work was largely supported by this grant—we apologize for the oversight)

*Enzyme Kinetics Model of the Bacterial Luciferase Reactions for Biosensor Applications, Y Feng, RH Davies, JD Andrade, Ibid., p. 88.

Abstracts:

*Bartholomeusz DA, Davies RH, Al-Sheikh YT, and Andrade JD, "Development of a Multi-Analyte ChemChip for Metabolic Assessment and Monitoring," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 231.

*Abernathy, JB, Davies, R, and Andrade J, ""Bioluminescence-Based Glucose Assay for a Multi-Analyte Biosensor," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 227.

Davies, RH Andrade, JD, "Diabetes-Related Luminescent Assays for Multi-Analyte Measurement" in *Diabetes Technology & Therapeutics*, 2003, **6**(2): p. 237

Abstracts Submitted:

Intern. Conf.on Bio- and Chemi-Luminescence, Yokohama, Sept. 2004:

Bartholomeusz DA, Davis RH, Andrade JD Method for implementing bioluminescence-based analytical assays in nanoliter volumes

Al-Sheikh Y, Abernathy J, Bartholomeusz DA, Davis RH, Andrade JD Multi-parametric experimental design of bioluminescence-based analytical assays

Theses in Preparation:
R Davies, PhD., Dec., 2004: Luminescent Assays for the Assessment of Metabolism and Renal Function: Towards a Multi-Analyte Biosensor.
D. Bartholomeusz, PhD., Spring, 2005: Fabrication of a Bioluminescence-Based Multi-Analyte Biosensor
X.Y. Yang, PhD., June, 2006: Homogeneous Chemiluminescent Immunoassay for Application to ChemChip Devices
Y. Al-Sheikh, PhD., Spring, 2005: Calibration, Data, Visualization, and Interpretation of a Multi-Analyte, Luminescence-Based ChemChip
P. Mohan, MSc., Spring, 2005: Toward a renal chip for assessment of kidney function
J. Jensen, PhD., Dec., 2006: Toward Analysis of Major Steroid Hormones via Personal Multi-analyte Biosensors: Patient Management of Endocrine Disorders.
M. Yang, PhD., June, 2007: Process and Fabrication Issues in the Preparation and Production of ChemChips

Proposal

OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.

1. TITLE OF PROJECT
Multi-Analyte Micro-Devices for Biomedical Applications

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," state number and title)
Number: PAR-01-057 Title: Technology Development for Biomedical Applications:Phased Innov (R21/R33)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR		New Investigator ☒ No ☐ Yes
3a. NAME *(Last, first, middle)* Andrade, Joseph D		3b. DEGREE(S) PhD
3c. POSITION TITLE Distinguished Professor		3d. MAILING ADDRESS *(Street, city, state, zip code)* Dept. of Bioengineering Univ. of Utah 50 So Campus Center Dr Rm 2480 MEB Salt Lake, UT 84112-9202
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Bioengineering		
3f. MAJOR SUBDIVISION College of Engineering		
3g. TELEPHONE AND FAX *(Area code, number and extension)* TEL: 801-581-4379 FAX: 801-585-5361		E-MAIL ADDRESS: joeandrade@uofu.net

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt ☐ No ☐ Yes If "Yes," Exemption No.	5. VERTEBRATE ANIMALS ☒ No ☐ Yes	
☒ No ☐ Yes	4b. Human Subjects Assurance No. 1ORG0000072 M1082-01	4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes	5a. If "Yes," IACUC approval Date 5b. Animal welfare assurance no A3031-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From: 05/01/02	Through 04/30/06	7a. Direct Costs ($) 99,633	7b. Total Costs ($) 149,450	8a. Direct Costs ($) 605,998	8b. Total Costs ($) 900,527

9. APPLICANT ORGANIZATION	10. TYPE OF ORGANIZATION
Name UNIVERSITY OF UTAH Address 1471 E FEDERAL WAY SALT LAKE CITY UT 84102-9020	Public: → ☐ Federal ☒ State ☐ Local Private: → ☐ Private Nonprofit For-profit: → ☐ General ☐ Small Business ☐ Woman-owned ☐ Socially and Economically Disadvantaged
	11. ENTITY IDENTIFICATION NUMBER 1876000525A1 DUNS NO. *(if available)* 00905365
Institutional Profile File Number (if known) UNKNOWN	Congressional District 2nd

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE	13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Vincent A. Bogdanski Title Manager, Grants and Contracts Address 1471 Federal Way Salt Lake, UT 84112 Telephone 801-581-3008 FAX 801-581-3007 E-Mail ospawards@osp.utah.edu	Name Lynne U Chronister Title Director, Office of Sponsored Projects Address 1471 E Federal Way Salt Lake City UT 84102-9020 Telephone (801) 581-3003 FAX (801) 581-3007 E-Mail ospawards@osp.utah.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE:	SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*	DATE
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.		09/27/01
15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* U Bogdanski Signing for	DATE 9/28/01

PHS 398 (Rev. 05/01) Face Page Form Page 1

Principal Investigator/Program Director *(Last, first, middle):* Andrade, Joseph D

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

We propose to develop microdevices for the specific chemical analysis of multiple metabolites in small sample volumes of biological fluids. The specificity and sensitivity is provided by specific reactions that couple analytes to bioluminescent-based enzyme reactions and produce light proportional to the analyte concentration. Bioluminescent analytical assays, in a miniaturized and stable format, can measure sub-microMolar concentrations in microliter sample volumes.

The goal of the first phase (R21) is to engineer microfluidic structures, develop enzyme packaging and stabilization techniques, and optimize optical detection systems in order to measure two model analyte solutions (galactose and lactate) using bioluminescent reactions.

The goal of the second phase (R33) is to implement other bioluminescent assays in the microfluidic detection system, develop specific diagnostic panels, utilize practical biofluid samples and enhance analytical accuracy and precision.

The proposed Micro-Analytical System (μAS) will be convenient to operate in point-of-care (POC) and home environments. It will likely evolve to measure up to 100 different metabolites in the sub-microMolar to milliMolar range from one 1-100 μL biofluid sample, and include customized comprehensive diagnostic panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs.

A schematic of the significance, proposed research, tools used, and future implications of the research can be seen in Figure 1 on page 13.

PERFORMANCE SITE(S) *(organization, city, state)*

Department of Bioengineering
College of Engineering
University of Utah
Salt Lake, UT

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Andrade, JD	University of Utah	Principle Investigator
Kern, SE	University of Utah	Co-Investigator
Miller, M	University of Utah	Co-Investigator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

Principal Investigator/Program Director *(Last, first, middle):* Joseph D. Andrade

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.
Type density and size must conform to limits and specifications provided in the PHS 398 Instructions.

RESEARCH GRANT

TABLE OF CONTENTS

* SBIR/STTR Phase I applications: Items A-D of the Research Plan are limited to 15 pages.

Appendix *(Five collated sets. No page numbering necessary for Appendix.)* ☐ Check if Appendix is included

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list):

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format (on preceding page) for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Steven E. Kern	Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Cornell University, Ithaca NY	BS	1983	Mechanical Engineering
Pennsylvania State University, Univ. Park, PA	MS	1986	Bioengineering
University of Utah, Salt Lake City, UT	Ph.D.	1995	Bioengineering

A. Positions Held

University of Utah, Salt Lake City, Utah
Asst Professor, Dept of Pharmaceutics/Pharmaceutical Chemistry. 2000-present
Asst Professor, Department of Anesthesiology, 1999-present
Research Asst Professor, Department of Bioengineering, 1996-Present.
Research Asst Professor, Department of Anesthesiology, 1996 - 1999.

Product Development Manager, SensorMedics Corp., Los Altos, CA 1995 - 1996.
Research Asst. Dept of Anesthesiology, Univ of Utah, Salt Lake City, Utah 1990-1995
Development Engineering Manager, C.R. Bard, Inc., North Reading, MA 1985-1990

Academic Awards
1. Research Award, Society for Intravenous Anesthesia, 1994.
2. Meritorious Award, Cost-Effectiveness in Anesthesia Awards Program, 1993, Zeneca Pharmaceuticals and Anesthesiology News.
3. Becton-Dickinson Graduate Fellowship Award, 1990, Dept of Bioengineering, University of Utah.

B. Selected Publications

1. Shafer SL, Kern SE, Stanski DR: The Scientific Basis of Infusion Techniques in Anesthesia. Published by Bard MedSystems Division, CR Bard, Inc. 1990.
2. Kern SE, Westenskow DR, Orr JA, Johnson JO: An effectiveness study of a new piezoelectric sensor for train-of-four measurement. Anesth Analg 1994; 78:978-82.
3. Kern SE, Johnson JO, Westenskow DR, Orr JO: A comparison of dynamic and isometric force sensors for train-of-four measurement using submaximal stimulation current. J Clin Monit 1995; 11:18-22.
4. Kern SE, Westenskow DR: Pharmacokinetic-based mini bolus delivery as an alternative to continuous infusion for drugs that exhibit a biophase lag. J Pharmacokinet Biopharm, 1997; 25:191-208.
5. Kern SE, Westenskow DR: Fuzzy logic for model adaptation of a pharmacokinetic-based closed loop delivery system for pancuronium. Artificial Intelligence in Medicine, 1997; 11:9-31.
6. Kern SE, Johnson JO, Orr JA, Westenskow DR: Clinical analysis of the flexor hallucis brevis as an alternative site for monitoring neuromuscular block from mivacurium. J Clin Anesth, 1997; 9:383-387.

Paper continued:

7. Lu JK, Manullang TR, Staples MH, Kern SE, Bailey PL: Maternal respiratory arrests, severe hypotension, and fetal distress after administration of intrathecal sufentanil and bupivacaine after intravenous fentanyl. Anesthesiology 1997; 87:170-172.
8. Johnson JO, Kern SE: Bispectral electroencephalographic analysis for patient monitoring during anesthesia. Advance in Anesthesiology, 1998; 16: 61-78.
9. Bailey PL, McJames SW, Cluff ML, Wells DT, Orr JA, Westenskow DR, Kern SE: Evaluation in volunteers of the VIA V-ABG automated bedside blood gas, chemistry, and hematocrit monitor. J Clin. Monit Comput 1998; 14:339-346.
10. Johnson KB, Kern SE, Hamber EA, McJames S, Gong G, Kohnstamm K, Egan TD: The influence of hemorrhagic shock on remifentanil: a pharmacokinetic and pharmacodynamic analysis. Anesthesiology, 2001, 94:322-32.
11. Kern SE, Fragen RJ, Fitzgerald PC, van Zeeland M, Johnson JO: Impact of rocuronium and pancuronium interactions on the maintenance of neuromuscular block during surgery. Can J Anesthesia. 2001, 48:129-32.
12. Burns TA, Shomaker TS, Patel BCK, Crandall A, Pace NL, Kern SE, Satovick NJ, Meyfroidt GJP: A Comparison of Oral Transmucosal Fentanyl Citrate, and Intravenous Fentanyl Citrate for Perioperative Sedation/Analgesia for Cataract Surgery. Am J Anesthesiology 2001; 28: 15-19.
13. Kern SE, Linares, JO: On evidence for low complexity in the electroencephalograph signal (EEG) during anesthetic concentrations in human. Submitted to Journal of Complexity, under review.

Research Support:

1. PI: Joseph D. Andrade, Ph.D. 7/97 - 6/00 Effort 10%
   Whitaker Foundation $170,962
   Personal Sensors for the diagnosis and management of metabolic disorders.
   This study sought to design disposable sensors for monitoring analytes using a unique bioluminescent assay technique Status: Complete
2. PI: Talmage D. Egan, M.D. 1/98 - 12/98 Effort 20%
   Foundation for Anesthesia Ed. & Research $25,000
   Quantitative pharmacodynamic analysis of hypnotic and opioid interactions in volunteers.
   This study investigated the interaction of anesthetic drugs on blocking noxious stimuli in volunteer subjects. Status: Complete
3. PI: Steven E. Kern, Ph.D. 9/98 - 9/02 Effort 15%
   Cognetix, Inc. Salt Lake City, UT $70,000
   Dose-effect relationship for novel peptide compounds derived from conotoxins.
   This study is determining the pharmacologic response to a class of novel acetylcholine blocking agents that are peptides derived from snail venoms. Status: On going
4. PI: Steven E. Kern, Ph.D. 1/00 - 12/00 Effort 15%
   Anesta, Inc. $75,000
   Analysis of novel OTS delivery of new pharmacologic compounds.
   This study evaluated the pharmacokinetics and pharmacodynamics of an oral transmucosal drug delivery system in human volunteers. Status: Complete
5. PI: Steven E. Kern, Ph.D. 11/99 - 10/00 Effort 5%
   Whitaker Foundation / National Science Found. $24,800
   Healthcare Technology, Economics, and Policy: an Evolving Balance - Cost Reducing Health Care Technology Symposia at the 2000 AAAS Meeting. Status: Complete.

Research Plan – R21 Phase

A. Specific Aims – R21 Phase

General Objective

Our goal is to engineer microfluidic structures, develop enzyme stabilization and packaging methods, and optimize optical detection systems that will measure two model analyte solutions (galactose and lactate) through standard bioluminescent detection assays with picomole sensitivity. Reagents will be prepared in dry, stable forms within microfabricated structures. Sample fluid delivery and mixing properties with the stabilized reagents will be studied, optimized and tested. Low cost optical detection systems for measuring light signals from the analytical arrays will be employed. An integrated micro analytical system (μAS) prototype will be developed and tested.

Specific Aims

1. Detection Limits and Scale Optimization - Design microscale chambers of various shapes and sizes and determine detection limits for specific bioluminescent analytical assays.

   Bioluminescent analytical assays will be prepared for measuring model analyte solutions ranging from 1nM to 1mM. Light from bioluminescent assays in square micromolded structures will be measured using a photomultiplier tube (PMT) luminometer and a charge-coupled device (CCD) camera detection system. Microstructures will range from 10pL-100μL in volume to determine detection limits. Improvements in detection limits will be investigated using micromachined optical enhancement features such parabolic chambers with reflective coatings. A light emitting diode (LED) luminescent standard will be developed to calibrate and compare detection systems. Concentration calibration curves showing the accuracy and resolution of detection methods will enable quantitative comparison between different well and detector designs.

2. *Reagent Packaging and Sample Delivery* - Engineer methods for packaging dry reagents in microfabricated chambers. Analyze transport, mixing, and diffusion characteristics of sample liquids with the dried reagents.

   Reagents in liquid, gel, or immobilized in polymeric bead form will be deposited in microfabricated wells using various techniques. The reagents will be freeze-dried and subsequently sealed in the wells. Reagent activity and stability will be investigated. Rehydration and mixing efficiency of liquid samples flowing into microfabricated chambers with lyophilized reagents will be determined. Surface modification of the channels and chambers will be explored in order to enhance mixing/delivery of samples. Control of sample volume will also be addressed in this aim.

3. *Detector Optimization* - Optimize photodetector selection, design, and coupling of luminescent reaction chambers to the photodetector by comparing signal characteristics of various detectors such as a CCD, complimentary metal oxide semiconductor (CMOS), linear diodes (including avalanche photodiodes), and custom arrays of photodiodes and photoconductors.

   Since high sensitivity is required for our device but spatial resolution is not critical, comparing detection limits of different photodetectors and optical coupling schemes will aid in designing a smaller, more efficient detection system for the final μAS device. Custom photodetectors will be fabricated according to the optimal reaction chamber dimensions and designs determined in the previous specific aims. Detector arrays will be built such that individual pixel(s) collect the maximum amount of light emitting from each well.

B. Background and Significance – R21 Phase

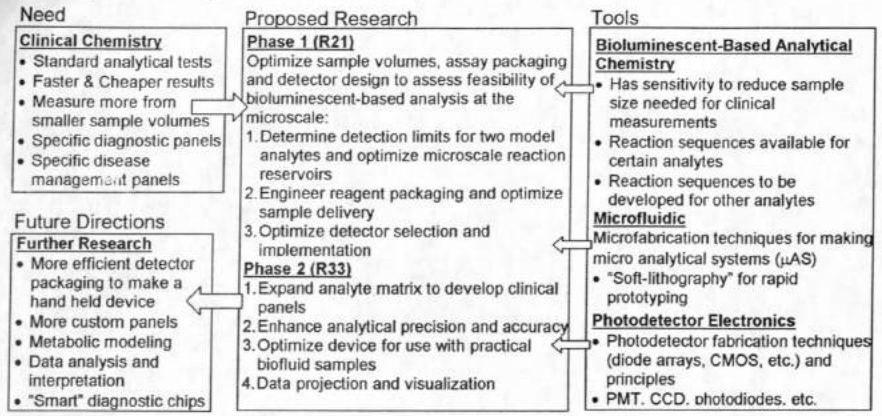


Figure 1: The Proposed Research: what need it fills, what existing tools and technologies will be used, and the future directions the proposed research will likely lead to.

Current Biosensing Methods

Advanced Point-of-Care (POC) biosensors are able to reduce metabolic analysis time, cost and sample volume for measuring key analytes (cholesterol, lactate, glucose) that usually exist in the milliMolar concentration range. These biosensors are generally not sensitive enough for analyzing other important metabolites that exist in the micro to sub-microMolar range. Current POC biosensors utilize enzymatic, electrochemical, and optical (fluorescence and absorbance) methods to detect various analytes [1]. Bioluminescence is 100 to 1000 times more sensitive than conventional fluorescence, which is a commonly used, highly sensitive analytical method. Bioluminescence assays have a detection range of five or more orders of magnitude without dilution or modification of the sample fluid [2, 3, 4, 5, 6, 7].

Proposed Biosensing Method

Bioluminescent-based chemical analysis has the potential to measure a wide range of metabolites from sample volumes that are smaller than those currently used in clinical chemistry. Since most metabolites in the body are within one or two enzymatic reactions from ATP or NADH [8], they can be measured by coupling the appropriate enzyme reaction(s) to an ATP or NADH bioluminescent reaction and measuring the light output. During the production or consumption of a metabolite of interest, enzyme linked reactions will cause the production or consumption of ATP (or NADH) through the bioluminescent platform reactions shown below.

The ATP platform is based on the firefly luciferase (FL) reaction:

(1) $\text{Luciferin} + \text{ATP} \xrightarrow{\text{Firefly Luciferase}} \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{PP} + \text{light (560nm)}$

The NADH platform is based on NADH:FMN oxidoreductase (OR) and bacterial luciferase (BL):

(2) $\text{NAD(P)H} + \text{FMN} + \text{H} \xrightarrow{\text{NADH:FMN oxidoreductase}} \text{NAD(P)}^{+} + FMNH_2$

(3) $FMNH_2 + \text{RCHO} + O_2 \xrightarrow{\text{Bacterial Luciferase}} \text{FMN} + \text{RCOOH} + H_2O + \text{light (490 nm)}$

Substrates are coupled to these platform reactions through the following generic reaction:

(4) Substrate + ADP (or NAD(P)$^+$) $\xleftrightarrow{\text{Enzyme}}$ Product + ATP (or NAD(P)H)

The changes in light intensity reflect stoichiometrically proportional changes in concentration of the platform molecule (ATP or NADH) and therefore, these light intensity changes are proportional to the metabolite of interest. These platforms have already been used by our group and by others to measure metabolites such as phenylalanine [9], glucose [10], glucose 6-phosphate [11], galactose [12], galactose-1-phosphate (G-1-P) [13], lactose [14], lactate [15], pyruvate [15], creatine, and creatinine [16] in solution, human blood (serum & plasma), and urine [17]. The many metabolites that can be measured via the ATP and NADH bioluminescent-based platforms make it feasible to incorporate several different analyte tests onto one device [18].

The increased sensitivity and dynamic range characteristic of bioluminescent detection is due to the high signal to noise ratio intrinsic to luminescence measurements and also because of the ability to "tune" the dynamic range via modulation of enzyme activity and/or enzyme type. This high sensitivity means that only small sample volumes are needed and that many different metabolites can be measured concurrently. It should be possible to measure 10 to 20 different analytes in the 10-microliter volume now used to measure only glucose with commercial glucometers.

Although bioluminescence analysis is well known and has been used regularly in research, it has not been widely applied to POC or routine clinical analysis for several reasons. Bioluminescence is able to detect trace concentrations, but requires highly sensitive, and therefore relatively expensive, luminometers. This limits the application of bioluminescence to those problems where such sensitivity is required, and therefore this technique is not seriously considered for the measurement of analytes in the microMolar to milliMolar range. Also, the luciferases and other reagents involved have developed a reputation for being somewhat labile, unstable, and difficult to utilize, with precise and somewhat sophisticated protocols. However, with recent advances in stabilization techniques, and the availability of highly active, thermally stable mutant luciferases we have the ability to prepare and stabilize many bioluminescent detection assays with relatively straightforward protocols [11, 19, 20, 21, 22].

Through careful integration of existing technology in enzyme stabilization, microfluidic fabrication, and optical detection systems, bioluminescence-based analysis can be successfully applied to μAS devices for POC applications. Passive microfluidic systems molded in polymers that can be sealed at low temperatures can package small amounts of enzymes, control sample flow and couple individual light producing chambers to separate photodetectors.

Proposed Microfabrication Method for Enzymatic Reactions in Small Volumes

We propose to use "soft-lithography" to create microfluidic chambers with optical enhancement features in micromolded PolyDimethylsiloxane (PDMS). PDMS will be used due to its modifiable characteristics and rapid prototyping capabilities [23, 24, 25]. High profile microchannels and chambers/wells of various shapes and sizes will be micromolded in opaque PDMS in order to determine the optimal well dimensions that maximize signal to sample volume ratios. Open wells will be molded, allowing enzymes to be deposited in patterns by discontinuous wetting [26], ink jet deposition [27], or in immobilized form within polymeric beads or agorose gels [28]. Glass or a layer of clear PDMS can seal the wells and couple them to light detectors. Such detectors include a single CCD camera or other imaging systems that are less expensive than PMT detection schemes, making the overall device more practical for POC application. Reflective metals can be patterned in the PDMS wells to create reflective surfaces for light collection enhancement [29]. By modification of the inert hydrophobic PDMS, we can control passive sample transport by permanently or temporarily rendering the walls hydrophilic [28]. Other chemicals can also modify the PDMS to minimize protein binding, which may become an issue when testing biological fluids

in the R33 phase [28]. This approach of fabricating and sealing the channels will avoid other bonding methods that often involve high temperatures (anionic bonding, metallic sputtering), caustic chemical bonding, or UV curing that can easily denature the deposited enzymes [30]. The ability to package and stabilize multiple assays in a microfluidic device would enable other enzyme-based analytical methods to be integrated on the same μAS device. Figure 2 shows a schematic of the proposed fabrication and testing procedures.

Applications

The high sensitivity and wide range of analytes that can be determined by bioluminescence using a single detection system make bioluminescence a practical detection method at the POC. Such a device could be used for basic and clinical research, personal disease management, or clinical and hospital use. It would provide rapid results, patient empowerment and reduce health care costs. Improved practicality to measure multiple metabolites at the POC would further increase the demand for understanding the complex relationships between diseases and their manifestation in the metabolic domain. Comprehensive metabolic diagnostic panels could be customized using existing knowledge of how certain diseases are manifested in abnormal metabolite concentrations. One example would be a low cost comprehensive inborn metabolic error diagnostic panel that can identify many disorders such as phenylketonuria (PKU) or galactosemia. Other panels can be developed as the complex metabolic relationships are discovered for certain diseases. This device could aid in collecting data for metabolic modeling, which will lead to understanding the complex relationships between diseases and metabolite concentrations.

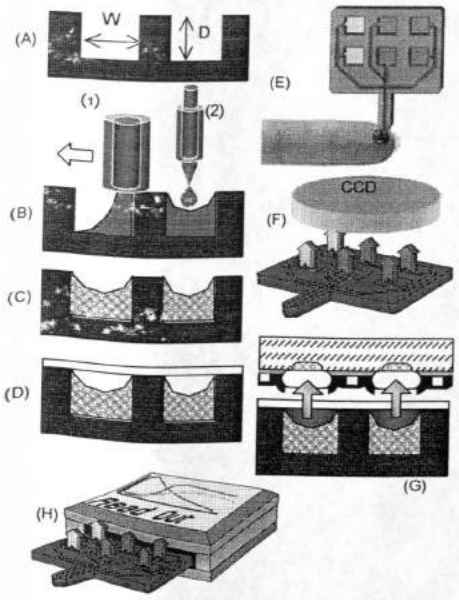


Figure 2: Schematic of Fabrication and Testing Procedures for Biomedical Microdevice

A) Microfluidic wells molded in opaque PDMS. Well width (W) ranges from 20-10,000μm and well depth (D) ranges from 20-1,000μm, giving well volumes ranging from about 10pL-100μL in order to test detection limits. (Detection Limits, pg. 16)

B) Separate bioluminescent assays (in solution or gel) for each analyte deposited by (1) discontinuous wetting or (2) ink jet deposition. (Specific aim 2, pg. 23).

C) Assays in buffer or gels are lyophilized (freeze-dried) for long-term stabilization (Lyophilization Procedures, pg. 24).

D) Glass or clear PDMS seal wells (Sample Delivery & Mixing, pg. 23).

E) Sample delivery by capillarity in modified PDMS (Sample Delivery, pg. 23). R21 phase - standard solutions. R33 phase - urine and blood.

F) Imaging detectors simultaneously detect individual well signals (Detection Limits, pg. 21).

G) Individual detectors sized to the optimal well dimensions and built in array in order to capture the most light from each well (Specific aim 3, pg. 25). Example shown is an illustration of integrated photodiodes in CMOS [31].

H) Concept of custom analyte panel with handheld detector.

C. Preliminary Studies – R21 Phase

Detection Methods and Detection Limits

Routine clinical chemistry assays have the potential to be measured with sample volumes in the picoliter to microliter range. Figure 3 shows the detection limits of various analytical methods using small sample volumes. Fluorescence, under optimal conditions with highly sensitive detectors and fresh reagents, has a detection limit of 1-femtomole (10^{-15}-moles) [32]. This means that a 1-femtoMolar solution is detectable from a 1-Liter sample, or a 1-nanoMolar solution from a 1-microliter sample, etc. (line 1, Figure 3). Despite this possibility, many clinical assays still require milliliter sample volumes [33].

Under optimal conditions, some luminescent-based detection assays are up to 1,000 times more sensitive than fluorescence (line 2, Figure 3)[2, 3, 4]. Luminescence therefore, has the potential to detect at a sensitivity of 1-attomole (10^{-18}-moles). This means that a 1-attoMolar solution is detectable from a 1-Liter sample, or a 1-nanoMolar solution from a 1-nanoliter sample, or 1-picoMolar solution from a 1-microliter sample, etc (line 2, Figure 3).

The sensitivity of luminescent-based analysis depends on the analyte and the quantum efficiency of the reaction. The 1-attomole bioluminescent detection limit described above is for the ATP/Firefly Luciferase reactions, which has a quantum efficiency (QE or ϕ) of 0.88. Reactions with lower quantum efficiencies would be less sensitive. For example, NADH/ Bacterial Luciferase coupled reactions have a QE of about 0.05 to 0.15 and would therefore have a sensitivity of 6 to 18 attomoles, which is still 50 to 100 times more sensitive than fluorescent-based analysis. This means that optimal luminescent-based analysis should be able to quantify analytes from sample volumes 50-150 (or even 1,000) times less than what would be required for optimal fluorescent detection. In order to accomplish this, the luminescent assays need to be correctly packaged in microfluidic channels that accurately and efficiently control the sample volume. Specific aim 2 will address sample volume delivery and control using microfluidic devices.

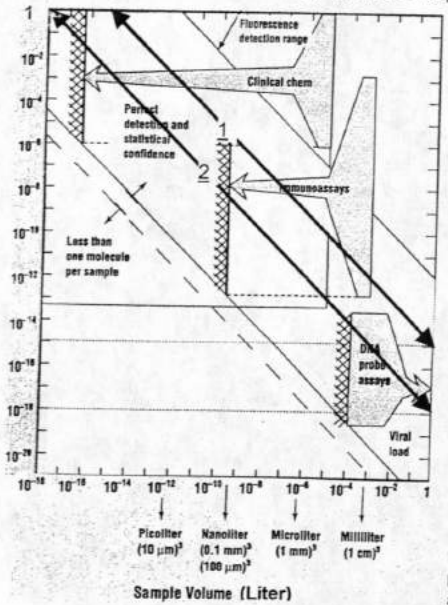


Figure 3: *Modified from Petersen et al.* [32] – Detection Limits Based on Sample Size and Concentration.

Detection limits in moles is the product of the sample volume (L) and the sample concentration (Molar) and is indicated by the diagonal lines. Although not shown in this graph, many clinical assays still require milliliter sample volumes.

Line 1 indicates the detection limit of highly optimized fluorescent-based analysis.

Line 2 indicates the detection limit of bioluminescent-based analysis with wet assays and PMT luminometers.

The choice of detector is critical in making luminescent-based analysis practical for POC use or as an inexpensive biomedical research tool. Most luminometric reactions are detected by PMT luminometers. PMTs have a high signal amplification with very low signal to noise ratios. These detectors cost over $50,000. Also, PMT is only able to detect light from a single source and would not spatially distinguish different analyte

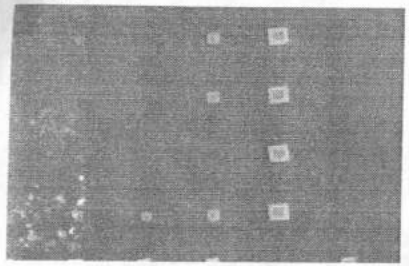


Figure 4: 20-Sec Integration of Bioluminescence in Anisotropically Etched Wells 250-μm Depth.

Silver coated wells. 750, 500, 400, 300, and 250 μm wide squares are visible. Note the enhanced light collection from the reflective 54.7° walls compared to the center of the wells. Measurements were taken using a 2-dimenitional ST6-A CCD camera by Santa Barbara Instruments Group.

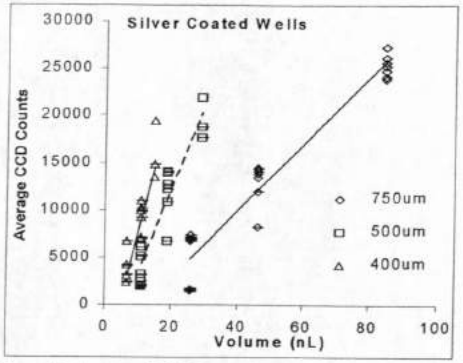


Figure 5: Average CCD Counts from the 20-Sec Integrated CCD Reading for Bioluminescence in Micro Reaction Chambers with Silver Coatings.

Data was plotted in sets for the same viewing area (or width of micro reaction chamber). For each set of data (wells with same width and coating), the increasing volume occurs from the three etch depths used (250-mm, 100-mm and 50-mm). The higher intensity values occur for the deeper wells. The intensity/volume slope indicates the effectiveness of increasing intensity with slight increases in volume.

channels in our micro analytical device. Although not as sensitive and having a signal gain less than 1, cooled CCD cameras capable of detecting low levels of light cost 90% less than PMT luminometers. The imaging capability of a CCD will allow multiple luminescent reactions for separate metabolites to be measured at the same time. The lower cost of the CCD and its ability to measure multiple metabolites at once will make the final cost and operation of the device more plausible to use at the Point-of-Care.

Our preliminary light intensity measurements of 1-mM ATP luciferase bioluminescent solution in simple microfabricated square wells (1 to 85-nL in volume) have shown the possibility of extending bioluminescent-based measurements to the microscale using CCD detection [34, 35]. Initial experiments using low volumes of ATP/Firefly Luciferase reactions were performed with a cooled CCD in order to estimate the signal limits. Figure 4 shows 1-mM ATP samples in silver coated pyramidal microwells etched in silicon. Figure 5 shows that the CCD counts from those wells ranged from about 5,000 to 25,000, depending on the volume of the sample (or etch depth). The total number of CCD counts per pixel (Nc) recorded for an image for the sample time (T = 20 sec), is related to the fraction of photons collected (E *for efficiency*), reflectivity of the surface (R_S), the collection angle (Ω = 2.18 sterradians for experimental set up in Figure 4) and photon flux (F) in (photons/sec/sterradian)), according to the equation:

Equation 1: $Nc = E \times \Omega \times F \times [1+R_S] \times T$. [36]

E is the product of the fraction of photons transmitted by the collection optics and CCD window (about 0.81 for the experimental setup), the quantum efficiency of the CCD (about 0.3 photoelectrons/photon for 560-nm wavelength of light and 0.1 for 420-nm)[37] and the ratio of CCD counts per photoelectron (0.15 count/photoelectron). From these numbers, E is calculated to be 3.6×10^{-2} CCD counts/(photon×pixel) for ATP reactions (560nm light) and 1.2×10^{-2} CCD counts/(photon×pixel) for NADH reactions (420nm light).

For the silver coated silicon wells, R_S at 560nm is = 0.86. The collection angle for this case may be greater than 2.18 sterradians because the well walls were angled to reflect and focus more light into smaller pixel areas. This increase in collection angle is assumed to be accounted for in the $[1 + R_S]$ term. Thus, the high volume (85nL) signals of Figure 5, where $Nc \approx 25{,}000$ CCD counts per pixel from the 250μm deep wells, F is about 8,500 photons/sec/sterradian for a 1mM ATP bioluminescent reaction.

The background noise for the 20-second experiments shown in Figure 4 and Figure 5, was 300 CCD counts/pixel. In order determine the lowest detectable ATP concentration using the CCD, we assume the minimum desired signal to noise ratio is 2. The lowest value for Nc would then be 600 CCD counts/pixel. Thus, accounting for the collection angle (Ω), the photon flux rate for the 1-mM ATP solution, and the depth of the well (ℓ), the lowest detectable ATP concentration is (L):

Equation 2: $$L = \frac{600 CCDcounts}{pixel} \cdot \frac{pixel \times photon}{0.036 CCDcounts} \cdot \frac{\sec \times ster}{8{,}5000 photons} \cdot \frac{1mMATP \times 250 \mu mdeep}{\Omega \ell} \cdot \frac{1}{Time}$$

The 1mM ATP concentration multiplied by the depth of the well corresponds to the number of ATP molecules per viewing window area. In other words, it is the 2 dimensional concentration of light presented to the camera pixel produced by the 2-dimensional concentration of ATP molecules with respect to the viewing window. This number is multiplied by the inverse of the initial 8,500 photons/sec/sterradian in order to obtain the ratio of ATP molecules per photon. Assuming that the signal is above the shot noise level and above the dark current reading of the CCD pixels, noise will not increase as much as the signal will with time [37]. Thus we can improve signal to noise ratio (and enhance the detection limits) by longer exposure times. Exposure time should be under 3 minutes to provide rapid results for POC applications [38].

In addition to time, the detection limit can be improved by changing Ω, and ℓ. The limit for Ω is 4π sterradians. We can approach this number by modifying the shape of the well and changing the reflectivity of the well walls such that more light is focused on a smaller pixel area (such as a parabolic well – see specific aim 2). Figure 4 shows how the anisotropically etched angled walls reflect more light toward the camera. Any of the light that was traveling parallel to the CCD within the well was reflected back to the camera. This suggests that a parabolic well will enable the collection angle to approach 4π sterradians.

The optimal value for ℓ can be determined by calculating the effect of absorbance of light through the luminescent media. According to the Beer-Lambert Law, the transmittance of light through a media along a channel or chamber of length ℓ, is defined by the Beer-Lambert Law as $T = 10^{-Absorbance}$, where $Absorbance = \varepsilon_\lambda C_B(\ell - x)$, ε_λ is the Molar absorption coefficient (L/mol·cm) and C_B is the concentration (mole/L)[39]. If light is generated from within a homogeneous media, the transmitting light is integrated along the length it travels and the total light transmitted ($T_{Total(\ell)}$) is defined as:

Equation 3: $$T_{Total(\ell)} = \int_0^{\ell} 10^{-\varepsilon_\lambda C_B(\ell - x)} dx = \frac{(1 - 10^{-k\ell})}{kLn10},$$

where $k = \varepsilon_\lambda C_B$. Because k varys as a function of the concentrations of the enzyme constituents, we plot $T_{Total(\ell)}$ versus channel length ℓ (Figure 6) for various values of k. For each curve, there is a region where $T_{Total(\ell)}$ increases almost linearly with ℓ. The smaller k is, the longer the linear region before $T_{Total(\ell)}$ plateaus. Therefore, by increasing the length of the channel (or depth of chamber) in which the bioluminescent reaction is occurring, we can increase the intensity of the signal at the detector. This property is effective for increasing the signal as channel length increases, until absorbance dominates the transmittance.

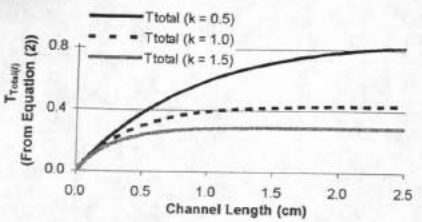


Figure 6: Total Transmittance of Light from a Homogeneous Light Generating Media Along Various Channel Lengths (ℓ) - At Various Values of k.

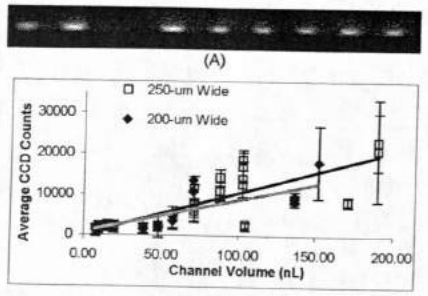


Figure 7: A) 11-mm long channels. Three 250-μm, and five 200-μm wide channels are seen. Difficulty in focusing the detector resulted in large errors in each of the measurements where the light boundary could not be easily determined.

B) Average CCD Counts for 250-μm and 200-μm Wide Channels at Various Volumes (proportional to Length)

Figure 7 shows an experiment where light was measured end on from long narrow channels of varying widths and lengths molded in PDMS (white opaque- RTV E, Dow Corning, Midland, MI)[35] filled with a 1mM ATP bioluminescent solution. The results show that light intensity increases linearly with channel lengths up to at least 12 mm, suggesting a low absorbance coefficient (or low k value in Equation 3). Thus absorbance isn't a factor for channel length if ℓ is on the order of 10 mm. One method of increasing the effective transmittance length ℓ of a micro-channel, without increasing volume, is to coat it with a reflective surface. A reflectance coefficient of 1.0 would essentially double the effective optical path without increasing sample volume.

The lower limit for ATP detection, L in Equation 2, can be determined by assuming numbers for ℓ, T and Ω. Assuming ℓ is 10,000-μm, a viewing window of 750-μm^2 (the well width that had the 25,000 CCD count signal), and assuming Ω approaches 4π, at an integration time of 100 seconds based on the discussion above, the lower limit of ATP detection using a CCD camera is a 39 nanoMolar solution of ATP from a 5.6 μL sample, or 220-femtomoles of ATP. Our estimate shows that the CCD is only $2x10^5$ times less sensitive than the PMT, while it should be over 10^6 times less sensitive. This is due to the longer signal integration time for the CCD, a principle that doesn't affect the PMT.

The actual detection limit will be above the estimated 220 femtomoles due to lower quantum efficiencies for combinatorial luminescent assays in their lyophilized form. Our previous studies concerning the effect of antioxidants on the stability of enzymes used for bioluminescent measurement of creatine showed that relative light activity is about 10% of that for the fresh, wet assays [40], for at least 60 days (see Figure 8). Assuming our detection sensitivity was 220 femtomoles, a 90% reduction in light output due to loss of enzyme activity would result in a sensitivity of 2.2 picomoles. The reduction in sensitivity that occurs for bioluminescent detection due to lyophilization and detectors with lower sensitivity would also occur in fluorescence detection if its reagents were lyophilized and less sensitive detectors were used. Thus, for comparison, lyophilized fluorescence with average cost fluorometers would have a sensitivity of 10-picomoles. Therefore, bioluminescence analytical methods still have the potential to measure lower concentrations from smaller sample volumes than fluorescence. Specific aim 2 will attempt to minimize the loss of activity due to lyophilization and determine the detection limits for lyophilized assays prepared within the microchannels. This will help assess the feasibility of implementing lyophilized bioluminescence detection in a μAS device for POC applications.

While the CCD detector used in the preliminary experiments described above had a gain (Q.E.) less than 1, other detectors with higher signal gains, such as avalanche photodiodes and photoconductors, can improve detection limits. These detectors can be built in arrays using standard semiconductor fabrication processes with each pixel sized to the optimal well dimensions in order to capture the most light from each well. The gain of some of these photodetectors range from 10^2 to 10^6 at room temperature [31]. Assuming only a gain of 10 to 1,000, we can increase the 2.2-picomole sensitivity of bioluminescent-based detection with lyophilized assays, to 2.2-220 femtomoles.

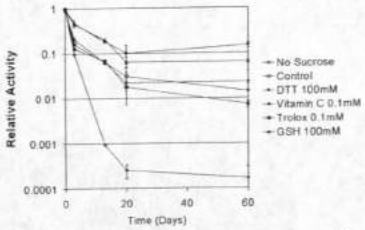


Figure 8: Stabilization of the creatine bioluminescent assay with different excipients. The activity is relative to a control firefly luciferase cocktail with no added antioxidant or sucrose before lyophilization. The error bars indicate one standard deviation [40].

In summary, the theoretical detection limit for bioluminescence detection of wet assays (specific aim 1) with a CCD is about 200-femtomoles. In specific aim 2, we will determine the detection limit for bioluminescence in its lyophilized form with CCD detection. This has been calculated to be 10 times higher than what is possible for specific aim 1, resulting in about 2-picomole sensitivity. The optimal detector selection to be determined in specific aim 3 should increase the sensitivity from specific aim 2 at least 10 fold. Therefore, if the theoretical detection limits for specific aims 1 and 2 are met, the detection limit with custom detector arrays should be about 200-femtomoles. If the theoretical 200-femtomole detection limit is attained, our proposed detection system would be able to detect 50 different analytes of 1-microMolar concentration from a 10-microliter sample, or measure one 2-nanoMolar analyte solution from a 100-microliter sample. The 1st phase of research will determine the actual detection limit for lyophilized bioluminescent assays at the microscale using analytes that we have previously studied: galactose [12] and lactate [15].

Table 1: Left – Various analytes and their approximate molar concentrations in the body. The lowest concentration we predict to measure from a 100 μL sample is 2-nanoMolar [41].

Type	Analyte	Body Concentration (Molar)
Electrolytes	Sodium	5.0×10^{-2}
Metabolites	Glucose	1.7×10^{-3}
	Cholesterol	1.3×10^{-3}
	Uric acid	1.3×10^{-4}
	Lactate	5.0×10^{-4}
	Creatinine	5.0×10^{-5}
	Galactose	2.0×10^{-5}
Hormones	Cortisol	1.3×10^{-07}
	Digoxin	1.3×10^{-09}
	Estrogens	6.6×10^{-11}
Others	Specific antibodies	1.7×10^{-13}
	DNA fingerprinting	1.7×10^{-15}
	Cancer detection	1.7×10^{-17}

Table 2: Below – Predicted theoretical detection limits for bioluminescent assays under various conditions that will be tested along with descriptions of fluorescent detection as a comparison.

The theoretical detection limit for specific aim 1 is 200-femtomoles. The theoretical detection limit for specific aim 2 is 10 times higher than that of specific aim 1 (2-picomoles). The theoretical detection limit for specific aim 3 is 10 times more sensitive than what will be possible for specific aim 2 (200-femtomoles).

Detection Methods	Assay Prep	Detector	Detection Limit (moles)	Volume Required to Detect a 1-microMolar Solution
Bioluminescence - Optimal	Wet	PMT	1×10^{-18}	1-pL
Fluorescence - Optimal	Wet	Fluorometer	1×10^{-15}	1-nL
Bioluminescence Specific Aim 1	Wet	CCD	2×10^{-13}	50-nL
Bioluminescence Specific Aim 2	Freeze-dried	CCD	2×10^{-12}	500-nL
Bioluminescence Specific Aim 3	Freeze-dried	Detector Array	2×10^{-13}	50-nL
Fluorescence	Freeze-dried	CCD	1×10^{-10}	100-μL

D. Research Design & Methods – R21 Phase

Specific Aim 1: Detection Limits and Scale Optimization - *Design microscale chambers of various shapes and sizes and determine detection limits for specific bioluminescent analytical assays.*

Instrument Calibration

PMT and CCD photodetectors need to be calibrated to the same light intensity units in order to accurately determine detection limits for microvolumes of luminescent fluids. Standard luminescent analysis using PMT luminometers is reported in relative light units (RLU), a unit applicable only to particular instrument setups. Detector calibration will be important in specific aim 3 in order to compare different detector schemes. Currently, most chemiluminescent and bioluminescent measurement systems are calibrated with luminol standards, which are prone to mixing variation and other errors. The luminol emission spectra is different than that of the firefly and bacterial bioluminescent assays. An ideal light standard would be a point source of low intensity, with approximately the same spectra for each bioluminescent platform of interest [42].

We propose to calibrate detectors with LEDs with emission spectra comparable to the firefly and bacterial luciferase reactions (which have peak wavelengths at 560 nm and 490 nm respectively). We will use surface mounted LEDs with an illuminated area of 250μm (AP2012, Kingbright Corp. CA) and peak wavelengths at 468 and 565 nm (±20nm FWHM) in order to approximate the dimensions of the micro wells that will house the bacterial and firefly luminescence reactions. Each LED will be supplied with constant current and the photon flux (photons/sec×sterradian) will be measured with an Oriel Photon Counting System (Model 76915, Oriel Instruments, CT) under the same optical setup that will be used for the CCD camera (ST7E, Santa Barbara Instruments, CA) and PMT luminometer (TD20-20, Turner Design, CA). CCD counts and PMT RLUs will then be correlated with the LED photon flux while accounting for the detector spectral response. The calibration experiments will be verified by comparing aqueous luminol standards (428 nm) with 430 nm surface mount LEDs. We will also verify firefly/ATP calibration standards [43] using 565 nm LEDs.

Detection Limits

Detection limits and calibration curves will be determined for both ATP/Firefly and NADH/Bacterial platform bioluminescent assays. ATP and NADH concentrations will range from 10-picoMolar to100-milliMolar in order to determine absolute detection limits for our proposed biosensor. Similar calibration curves and detection limits will be performed for galactose using the ATP platform [12], and lactate using the NADH platform [15] in order to show the feasibility of using luminescent based detection for two important model analytes. Calibration curves and detection limits will be compared with traditional fluorescence enzyme assays at similar sample volumes.

The goal of the galactose and lactate measurements will be to create calibration curves within their representative concentration ranges in blood. Blood galactose concentrations range from 20 to 90-microMolar [44], while blood lactate concentrations range from 0.5 to 2.5 milliMolar [15]. The error of the galactose and lactate measurements should not exceed those set forth by CLIA (Clinical Laboratory Improvement Amendments - 1988) analytical quality requirements [45].

Galactose will be detected by the ATP bioluminescent platform according to the following sequence:

(1) Galactose + ATP $\xleftrightarrow{\text{Galactokinase, Mg++}}$ Galactose-1-Phosphate + ADP

(2) Luciferin + ATP (decrease) $\xrightarrow{\text{Firefly Luciferase}}$ Oxyluciferin + AMP + CO_2 + PP + light (560nm)

Lactate will be detected by the NADH bioluminescent platform according to the following sequence:

(1) Lactate + NAD^+ $\xleftrightarrow{\text{Lactate Dehydrogenase}}$ Pyruvate NADH + H^+

(2) NAD(P)H + FMN + H $\xrightarrow{\text{NADH:FMN oxidoreductase}}$ $NAD(P)^+$ + $FMNH_2$

(3) $FMNH_2$ + RCHO + O_2 $\xrightarrow{\text{Bacterial Luciferase}}$ FMN +RCOOH + H_2O + light (490 nm)

Reagent and enzyme proportions will be created such that the light intensity after the first 5 to 10-seconds of mixing will be approximately constant and proportional to the analyte concentration. This will be accomplished by ensuring that analyte concentrations are much greater than luciferase concentrations [5] and verified with PMT luminometer measurements. A constant light output will help to normalize the integrative CCD counts with exposure time. This will also eliminate any errors associated with slow mixing and reduce the need to account for intensity fluxuations that would occur, as reaction chambers are being filled and covered with the glass slides.

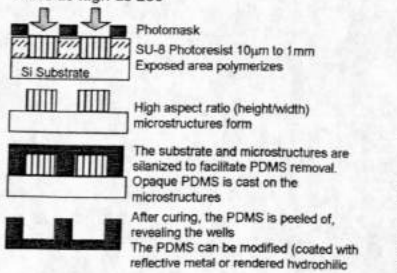
In order to test the detection limits for luminescent assays, we will fabricate reservoirs that range from 10pL to 100μL. We have had experience in micromolding square wells in PDMS [35], following "soft lithography" techniques demonstrated by *Whitesides, et. al.* [23,24]. This method is chosen for its ability to rapidly produce prototypes of deep wells with precise dimension control. Some groups have observed shrinkage of the PDMS after curing, but this occurs in non-polar organic solvents and any dimensional changes that occur due to curing can be verified and accounted for by measuring the final well dimensions with SEM measurements. The wells will be fabricated at widths of 20 to 10,000-micrometers and at depths of 20 to 1000-micrometers, creating volumes ranging from about 10-pL to 100-μL. By choosing these dimensions, we will be able to compare average light intensity for wells of the same volume but different depths and window sizes.

To create the micromold master, rectangular extrusions are patterned on a silicon wafer using a thick negative photoresist (SU-8) [46], which is capable of producing aspect ratios as high as 250 and feature sizes as tall as 1200-μm (Figure 9). The extrusions are then silanized [24] in order to facilitate the release of the mold. PDMS will be cast and cured on the mold masters. The PDMS mold becomes an inverse of the patterned extrusions, creating precise volumetrically controlled wells. Interconnecting channels can also be molded in PDMS. In order to prevent cross talk of light between reservoirs, we will use a black, opaque PDMS (Dowcorning, Sylgard 160). The wells and channels can be temporarily sealed by placing a clean glass cover slip in conformational contact with the PDMS. If a more permanent or stronger seal is required, we will oxidize the PDMS in a plasma cleaner (Oxford Plasmod) [24]. All micromachining and micromolding will be performed in the University of Utah's microfabrication facility by experienced personnel.

The reaction reservoirs will be filled by pipetting excess solution over a number of wells, covering it with a glass cover slip and pressing any excess fluid out from between the PDMS walls and the glass (Figure 10). Average light intensity from the wells will be measured using Scion Image. Background noise is measured as the average intensities of the dark areas surrounding the wells and is subtracted from the average intensities of filled wells in order to account for any thin films of luminescent solution that may still remain between the PDMS and the glass. This method will allow the viewing of light from volumetrically controlled wells in order to determine detection limits, without needing to design complex channels to fill the wells via capillarity. Although we have seen bubbles form in wells by this method [34,35], the occurrence is rare and those wells that have bubbles are easily seen in the CCD image and eliminated from measurements.

CCD measurements can be performed for multiple

Figure 9: Patterning of mold in thick photoresist. Depths can be from 10 to 1,200-μm with aspect ratios as high as 250



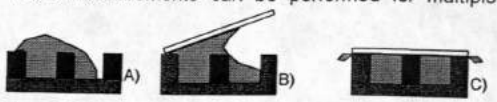


Figure 10: Flood filling of the microwells

A) Liquid is placed on the molded PDMS. B) A glass cover slip is applied at an angle, allowing the excess fluid to fill other wells and be squeezed out sides (C).

wells at the same time; however, we will need to compare this method with standard luminometer reading. In order to accomplish the low volume luminescent measurements in the luminometer, the windows over the wells will be masked such that only one well is open to the PMT detector. If this method still allows too much stray light, capillary tubes will be cut to exact lengths to produce reservoirs of the same volumes as those that will fabricated for CCD detection. The capillaries will be coated with black paint and lined up end on to the PMT to simulate the wells.

Light Collection Enhancements

As mentioned before, some of our experiments have shown that the light collection can be increased by the shape and reflectivity of the well. The brighter sides of the wells in Figure 4 shows how 54.7° angled walls coated with silver (R_S = 0.86) can enhance the light reflecting to the camera. This, in effect, increases the collection angle (Ω in Equation 1).

Parabolic reflectors can be molded in the wells to increase the collection angle. Kobayashi *et al.* has developed a method to create microscale lenses [47]. This is accomplished by patterning circular hydrophilic regions on a hydrophobic surface and then dipping the patterned surface into a hydrophilic polymer, which forms round beads that become polymeric lenses when cured. These beads become the mold master for rounded wells in PDMS, instead of the rectangular well molds as seen in Figure 9. We can apply this procedure to make molds of different parabolic dimensions. The PDMS can be coated with silver such that only the parabolic wells are coated [48]. The optimal parabolic shape (characterized by the focal point) will be a function of the distance from the CCD camera and the lens used to focus on the wells. Average intensities and detection limits will be compared to the experiments performed with the non-reflective square wells. Similar lenses used to create the mold masters for the wells can be patterned on the glass cover slip in order to further focus the light on the CCD.

Specific Aim 2: Reagent Packaging and Sample Delivery - *Engineer methods for packaging dry reagents in microfabricated chambers. Analyze transport, mixing, and diffusion characteristics of sample liquids with the dried reagents.*

The goal of this aim is to package homogeneous enzyme solutions within the wells that are stable for periods from six months to a year. After the optimal well dimensions and volumes are determined from specific aim 1, we will test lyophilized solutions for the measurement of ATP, NADH, galactose and lactate within sealed wells. Initially, we will fill the wells with buffered detection assays. Due to the small volume of the wells (10-pL to 100-μL), it might be difficult to fill them by ink-jet or syringe. Therefore, our first approach will be to use the discontinuous wetting approach by *Whitesides, et. al.*, which was able to fill pL to nL size wells of various dimensions in PDMS using a syringe pump controlled by an XY positioning stage [26]. This method works by dragging a wet "pen" over each well and the hydrophobic PDMS causes the fluid to pull into the well corners, effectively filling the wells (Figure 2 - B). These filled wells will be lyophilized using our Genesis 12EL lyophilizer, (Virtis, NY). After lyophilization, the well array will be covered with an oxidized clear PDMS or temporarily covered with a glass cover slip. The wells will be filled by methods described below. Luminescence will be detected by CCD as explained in specific aim 1. Calibration curves and detection limits will be repeated by varying analyte concentrations in the solutions.

Sample Delivery & Mixing

Sample fluid can enter the wells through the top, via channels molded in clear PDMS that are stacked and aligned with the wells using a channel layering technique demonstrated by *Beebe, et. al.* (Figure 11) [49]. This channel layer can be aligned to direct sample fluid to each well by capillarity. The exiting channel allows gas to escape. A layer of PDMS with channels can be placed on top or beneath the wells. To access the bottom of the wells, holes can be punched or molded into the opaque PDMS.

For simplicity of operation, the final device should be able to passively transport the sample fluid to the reaction chambers. The hydrophobic properties of PDMS could limit the capillary flow of the samples and produce non-uniform filling of the wells. However, we propose to overcome this challenge by oxidizing the PDMS thus rendering it hydrophilic [23]. However, this is only a temporary surface modification. We can treat the PDMS with a 5% methanol solution of PDMS-*b*-PEG (polyethyleneglycol)[28] for a more permanent modification. This method will even enhance gel solutions (before gelation) to flow through microchannels even after channels are exposed to air for 24 h. It may be necessary to fill the channels or wells with blank gels in order to facilitate sample flow in the channels or provide a stable matrix for enzymes to be immobilized to in the wells. Dehydrated gel would provide a microscopic capillary matrix for the sample fluid to flow through. The matrix size can be customized by the type of gel used.

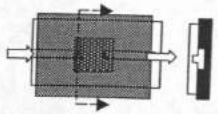


Figure 11: Clear PDMS channels leading to the wells molded in opaque PDMS

The channels molded in clear PDMS are aligned to the top of the wells (dark square), such that there is an inlet channel and an exit channel. The figure on the right shows a cross section of the clear PDMS with the channel and the opaque PDMS with the well.

Lyophilization Procedures

Our lab experience has helped us determine appropriate stabilizing agents and preservatives that minimize the reagent degradation that generally occurs during the lyophilization process [40]. We will use disaccharides and neutral, hydrophilic polymers in order to control the glass transition temperature, which should be greater than the final storage temperature of the lyophilized enzyme. The sugar to protein weight ratio should be at least 1 to 1, although stability can be further increased with greater sugar weight ratios (5 to 1) for some enzymes. Reducing sugars such as glucose, lactose, maltose or maltodextrins will be avoided because of their tendency to degrade proteins through the Maillard reaction between the carbonyls of the sugar and the free amino groups of the protein. Antioxidants such as dithiothreitol and glutathione will be used during lyophilization and subsequent storage to prevent oxidation of firefly luciferase through sulfhydryl groups. Bovine serum albumin can be used for surface passivation and as a stabilizing agent. Polyethylene oxide (PEO)-based polymers and surfactants are also effective for surface passivation.

At this time our bioluminescent reagents are preserved and stabilized with the following components:

1) 0.45 M glycyl-glycine buffer (pH 7.8)
2) 1 mM EDTA
3) 1mM dithiothreitol
4) 1 mg/ml bovine serum albumin
5) 10 mM $MgSO_4$
6) 1 wt% sucrose
7) 1 wt% Dextran T-40.

The bioluminescent reagents (ATP, FMN, bacterial and firefly luciferase, oxidoreductase, etc.) are mixed thoroughly with the preservatives and added to each well of the pre-chilled biosensor. The biosensor and reagents are flash frozen with liquid nitrogen followed by a two-stage lyophilization process. The first stage of lyophilization proceeds for 24 hours at -50°C and < 100 mTorr of pressure. The second stage of lyophilization proceeds for an additional 24 hours at +30°C and < 100 mTorr of pressure. If the insulator properties of the PDMS affect the temperature control of the reagents during lyophilization, we can use a thermally conductive PDMS (PN# 3-6642, Dow Corning, MI). Due to the microscale volumes of reagents in the microdevice, the lyophilization times will be much shorter than 24 hours. Small volumes will result in rapid evaporation of the solvents and should quickly fixate the enzymes in a stable glass. Air or nitrogen gas is then re-admitted to the lyophilization chamber and the biosensors are removed. Each completed biosensor is then stored in a black plastic container with a gas tight lid that also contains a desiccant and a humidity indicator membrane. Our experience to date with firefly luciferase indicates that this approach to preservation can preserve more than half the enzymatic activity for a minimum of six months [40].

Other Stabilization Methods

In biosensor applications, enzymes can be immobilized on a solid support to prevent diffusion (into the sample solution) and to minimize interference with other channels of the sensor. If the lyophilization procedures described above do not work for non-immobilized enzyme solutions, we will need to rely on previous experience with immobilizing enzymes within gel or polymers [12, 19]. Gel or polymer stabilization methods have been used at the microscale by micromolding in capillaries (MIMIC) [19, 28,25]. A variety of solid matrices can be used in immobilization [50, 51, 52]. Controlling the polymer proportions and subunit size can create gels of sufficient pore size that allow enzymes and reagents to diffuse into the matrix. The performance of the sensor can be adjusted by changing the immobilized enzyme amount. Immobilized enzymes can be stored in supersaturated conditions and thus provide higher luminescent intensities, possibly increasing the sensitivity and/or range of each assay.

Stabilization Studies

As successful calibration curves and detection limits are found for lyophilized assays, each assay will be stored in light tight packages with desiccant for 1, 3, and 6 month durations. At these times, the calibration curves and detection limits will be repeated. Continued stabilization studies for 12-month duration will continue in the R33 phase if the R21 milestones are met. Onboard calibration features will also be initially tested during these studies. For each lyophilization time trial, a series of identical bioluminescent assays will be tested with a range of concentrations for the appropriate analyte. The light measurements from each concentration will be compared to a reference concentration. The final device in R33 can include the reference solution within the device through proper packaging and can function as an onboard calibration feature.

Specific Aim 3: Detector Optimization - *Optimize photodetector selection, design, and coupling of luminescent reaction chambers to the photodetector by comparing signal detection characteristics of various detectors such as CCD, CMOS, linear diodes (including avalanche photodiodes) and custom photodiodes and photoconductors.*

Although the PMT luminometer and CCD detection systems are standard methods for measuring bioluminescent reactions, they are not optimal for microscale wells. PMT luminometers are the standard detection method for luminometric assays due to their high sensitivity, fast response times, and large detection range. They cannot measure signals from multiple wells. The pixilated resolution of the CCD permits imaging and therefore multi-well/multi-analyte detection. The integrative nature of the CCD limits the ability to follow rapid changes in light intensity, in part due to the serial nature of the pixel output. The frame reading rate can be enhanced by software and hardware or binning of the pixels, but high clock rates would not allow the CCD charged pixels to fill above their thermal noise level. The ideal detector for an array of luminescent micro assays would have the sensitivity, range and response time of the PMT with the imaging performance of a CCD. Although arrays of PMTs are indeed used for other applications, this would too expensive and too large to be practical for an inexpensive, home use or POC device.

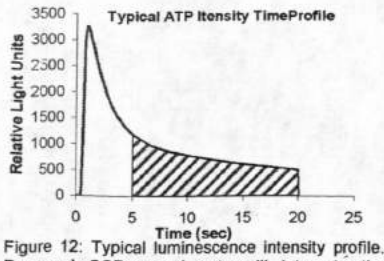


Figure 12: Typical luminescence intensity profile. Proposed CCD experiments will integrate the signal after the profile starts to level off. Active pixel arrays like CMOS detectors have fast response times that can detect the whole profile. Although some CCDs have fast frame rates that can detect the signal above, they are more expensive than the active pixel arrays we propose to test.

Other types of photodetectors can be fabricated in active pixel arrays with various dimensions, fast response times, and dynamic ranges greater than the CCD. These detectors include photoconductors, p-n & p-i-n junction based photodiodes, and avalanche photodiodes [31]. Photoconductors can be customized for specific bandwidths and don't have to be cooled. The gain of

these photodetectors can be as high as 10^7. The proposed photoconductor material is SiGe and CdS. Active pixel arrays can be built cheaper and with more processing circuitry than the CCD alternatives. CMOS, p-n, and p-i-n photodiode arrays also have higher frame rates, which will allow detection of the fast changing bioluminescent pulse (Figure 12). Variations in the pixel output of these arrays can be calibrated off-chip. Avalanche Photodiodes (APD) can also be fabricated in arrays. They have a larger gain (10^4-10^6) that p-n & p-i-n junction based photodiodes, but they do require high voltages and have high noise levels.

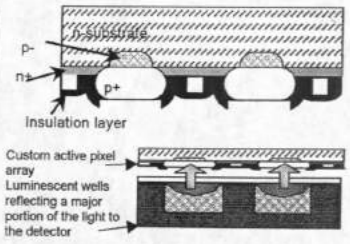


Figure 13: Top) Example of a proposed CMOS Custom active pixel array.

Bottom) Custom arrays that line up individual pixels with the entire luminescent well. Detectors can be in close contact with the well windows, reducing any loss of light that would occur with complex optical systems.

We will build individual detector arrays, sized to the optimal well dimensions in order to capture the most light from each well (Figure 13). Each pixel in the array will line up with the entire luminescent well. Detectors can be in close contact with the well windows, reducing any loss of light that would occur with complex optical systems. Instead of just one large (large being the size of the microwell) pixel per well, it may be beneficial to have 3-4 pixels per well in order to account for statistical variations between wells.

Using the micromachining facilities here at the University of Utah (the HEDCO Facility), we will fabricate and compare the detection limits of photoconductors, CMOS, p-n & p-i-n junction based photodiodes, and avalanche photodiodes. Each of these detection schemes will be designed to fit above an array of luminescent reaction wells having the dimensions determined from the experiments in specific aim 1. Detectors will be calibrated according to the methods described in specific aim 1. Dr. Miller will play an important role during the second year and into the R33 phase. He is currently the director of HEDCO and teaches the integrated semiconductor device class/lab, which covers photodiode arrays. He has experience with detector characterization and with CMOS design and fabrication.

Final experiments will integrate the optimized procedures and devices from all three specific aims. The optimal well designs determined from specific aim 1 will be filled with bioluminescent detection assays for measuring ATP, NADH, galactose and lactate. These assays will be prepared, lyophilized and covered using the most favorable techniques and designs determined in specific aim 2. The optimized detection system determined from specific aim 3 will be used to obtain final measurements. Final experiments will measure all four analytes in separate wells on the same microfluidic device from the same sample fluid containing various concentrations of the analytes. Calibration curves for each analyte will be repeated under these conditions.

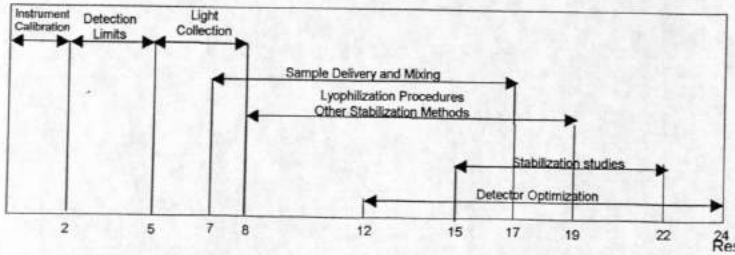


Table 3: R21 Timeline in months. 2 years total.

Milestones – R21 Phase

Specific Aim 1: Detection Limits and Scale Optimization - *Design microscale chambers of various shapes and sizes and compare their detection limits for specific bioluminescent analytical assays.*

The CCD and PMT luminometer will be calibrated to photons/(sec×sterradian) using the LED proposed light standard and luminol or ATP/firefly luciferase standards. If the limits of agreement between the calibration values from the LED and chemiluminescent standards show no difference between the two methods, within the 95% confidence interval, we will use the LED standard for calibrating the photodetectors [53]. Otherwise we will continue calibration curves with luminol standards.

The theoretical detection limit for CCD detection of fresh luminescent assays is 200-femtomoles. However, since many clinical analytes are only as low as 1-microMolar, and the highest volume we would hope to use for one analyte is 10-μL, the detection limit for ATP detection with the firefly luciferase luminescent platform should be at least 10-picmoles. The CLIA (Clinical Laboratory Improvement Amendments – 1988) quality requirement for many clinical assays is about 10% of the target value for many metabolites. The total error by the bias and the coefficient of variation should be less than one third of the quality requirement [38]. For galactose and lactate, the maximum allowable error is 10%, which means the total error of the bias and the coefficient of variation for both these tests should be 3.3% of their corresponding lower target range values. Because this phase is just the preliminary feasibility study we will first attempt to reach the 10% maximum error and address the higher precision issues in the R33 phase. Therefore, our approach should only have a 10% error for the detection limits and ranges described below.

Assay	Detection Range	Volume	Detection Limit	Maximum Error
ATP detection by FL reaction			10-picomoles	1-picomole
NADH detection by OR/BL reaction			200-picomoles	20-picomoles
Galactose by ATP/FL platform	20 to 90-microMolar	1-μL	20-picomoles	2-microMolar
Lactate by NADH/OR/BL platform	0.5 to 2.5 milliMolar	1-μL	500-picomoles	0.05-milliMolar

These detection schemes will be statistically compared to fluorescence assays as a way for comparison.

At the completion of this phase, we will have a matrix of well volumes and dimensions optimal for certain ranges of analyte concentrations. The optimal dimensions can be used for other analytes, whose desired detectable ranges will vary and will be investigated in the R33 phase.

Specific Aim 2: Reagent Packaging and Sample Delivery - *Engineer methods for packaging dry reagents in microfabricated chambers. Analyze transport, mixing, and diffusion characteristics of sample liquids with the dried reagents.*

Assuming the lyophilized assays will be about 10% as sensitive as the wet assays in specific aim 1. Therefore the lyophilized bioluminescence detection should have the following detection limits and errors:

Assay	Detection Range	Volume	Detection Limit	Maximum Error
ATP detection by FL reaction			100-picomoles	2-picomole
NADH detection by OR/BL reaction			2-nanomoles	40-picomoles
Galactose by ATP/FL platform	200 to 900-microMolar	1-μL	200-picomoles	4-microMolar
Lactate by NADH/OR/BL platform	5 to 25 milliMolar	1-μL	5-nanomoles	0.1-milliMolar

Stabilization studies should show that these detection limits and errors are achievable for assays stored at room temperature for as long as 6 months. The increased errors are 20% of the detection limits due to the expected increase in variability for lyophilized assays. Sample fluid mixing with lyophilized reagents should not affect the calibration range or resolution milestone projected for this specific aim.

The sample fluid must flow from an input port and fill each well without any technical difficulty or complicated pumps. The sample fluid must fill greater than 95% of the wells without bubbles. Bubble detection will be addressed in the R33 phase along with other quality assurance features.

Specific Aim 3: Detector Optimization - *Optimize photodetector selection, design, and coupling of luminescent reaction chambers to the photodetector by comparing signal detection characteristics of various detectors such as CCD, CMOS, linear diodes (including avalanche photodiodes) and custom photodiodes and photoconductors.*

The goal for choosing the optimal photodetector is to increase the sensitivity 10 fold from that of the CCD. Therefore, the milestone for this specific aim should improve the resolution and detection of lyophilized bioluminescent assays at least 10 fold from what is described in specific aim 2. Although sensitivity increases, the maximum errors are still expected to be 20% due to the increased coefficient of variation that is expected for the lyophilized assays. The R33 phase will address quality assurance and extend the stabilization studies in order to reduce the maximum error down to 3.3% of the target values. The detection limits and maximum errors for this phase should be as follows:

Assay	Detection Range	Volume	Detection Limit	Maximum Error
ATP detection by FL reaction			10-picomoles	2-picomole
NADH detection by OR/BL reaction			200-picomoles	40-picomoles
Galactose by ATP/FL platform	20 to 90-microMolar	1-μL	20-picomoles	4-microMolar
Lactate by NADH/OR/BL platform	0.5 to 2.5 milliMolar	1-μL	500-picomoles	0.1-milliMolar

Summary

If these detection limits are attained, bioluminescence will be a practical approach to measuring multiple analytes from small sample volumes. The detection limit of 10.0±0.3 picomoles for the ATP/Firefly Luciferase reaction would indicate that it is possible to measure 10 different analytes, each 10microMolar in concentration from a 100-μL sample divided and delivered into 10 different bioluminescent based analytical chambers. Should this milestone be met, the R33 phase will expand the number of analytes that can be measured, further optimize sample separation and delivery, and increase stabilization studies to at least 12-months. This extended research will also develop quality assurance features in order to reduce the coefficient of variation for each assay in order to make the maximum error less that one third of the analytical error quality requirements set forth by CLIA (3.3% of target for many analytes) [45].

Our work will be made widely known to the biosensor/analytical biochemistry/clinical chemistry communities via participation in conferences and publication in the appropriate journals, such as Analytical Biochemistry, Analytical Chemistry, Clinical Chemistry, Luminescence, and Biosensors and Bioelectronics. We will also expand our web site to facilitate communication with the interested communities. We are also beginning to work with patient groups who would benefit from such devices, including Parents of Galatosemic Children, PKU Parents (and their newsletter PKU News, etc.)

The University's technology transfer office is working with several companies who have expressed interest in this work. We now have one small industrial contract and expect several other small, feasibility contracts for specific analytes/sensors. This grant will enable the development of bioluminescence in the stable format at the microscale for model analytes, which can then be developed for specific clinical needs and potential commercial interests.

Research Plan – R33 Phase

Specific Aims – R33 Phase

General Objective

The goal of this phase is to expand the feasibility and optimization work in the R21 phase for the development and testing of other clinically useful multi-analyte microanalytical devices. In this phase, bioluminescent microfluidic assays will be developed for specific diagnostic and disease management panels. The devices will be tested, evaluated, and optimized for use with practical, clinically relevant biofluid samples, particularly blood and urine. Quality assurance issues will also be considered and studied. Indeed, we expect to develop a standard process with which to develop ATP and NADH bioluminescence-based analytical assays, including their lyophilization and evaluation in a dry reagent mode.

Specific Aims

1. *Analyte/Panel Development* - Develop bioluminescent assays for custom panels of specific clinical interest and relevance, including those appropriate to the management of several chronic metabolic diseases.

   Each assay will be optimized for the physiologic/pathologic concentration range needed for blood and/or urine samples. Extensive calibration and validation studies will be performed to fully optimize and evaluate each individual analyte assay. At this time the analytes we are most likely to include in the R33 phase include (in addition to galactose and lactate): phenylalanine, glucose, glucose 6-phosphate, galactose-1-phosphate (G-1-P), lactose, pyruvate, creatine, and creatinine. Others are likely to be added in response to the input of our clinical lab colleagues and our own work on metabolic modeling.

2. *Blood and Urine Samples* – Test and validate luminescent analysis with standard urine and plasma samples.

   Develop means to account for urine sample dilution on the device. Develop the means to perform the red cell separation from blood, using commercial plasma separation membranes on the sample inlet of the device. We will also facilitate total blood analysis, by incorporating hemolysis agents (in the absence of a plasma separation membrane) on the inlet channel of the appropriate section of the device. We will develop a hematocrit measurement in order to accurately interpret the blood and plasma measurements.

3. *Quality Assurance* - Minimize measurement errors and develop quality assurance features such as bubble/incomplete well filling detection, onboard calibration channels, and optimum or dual ranging detection.

   The methods and approaches here are similar to that developed for the R21 phase, but involves a larger number of analytes and channels. Develop means to expand analytical ranges for several analytes in order to be able to detect and compare normal and abnormal levels that may vary considerably in concentration. Extended stabilization studies will be performed. Develop means to reduce error by testing more stabilizers for lyophilization and including onboard standards.

4. *Data Analysis and Multi-Parameter Visualization* – Develop analytical algorithms to facilitate data presentation and usefulness; using the on board reference and calibration channels, a calibration model will be developed for each analyte and for each type of biological sample (blood, plasma, urine). For some analytes, ratios, differences, or other processing of the raw data will be required to produce a useful, interpretable output.

   We will experiment with multi-parameter visualization tools, particularly multi-axes radar plots to produce disease and condition specific signatures or patterns which can be made recognizable by a lay patient population.

Research Design & Methods – R33 Phase

The ability to rapidly detect multiple analytes from small sample volumes will make it feasible for doctors to obtain comprehensive multi-analyte information without jeopardizing the patient's comfort and without increasing health care costs. A 20 analyte metabolic panel will provide an initial comprehensive window to the patients' metabolic profile, his/her personal metabolic "metabolome". As the ability to read metabolic profiles becomes practical, there will be a need to expand the research in order to interpret the data. Improved practicality to measure multiple metabolites at the POC would further increase the demand for understanding the complex relationships between diseases and their manifestation in the metabolic domain.

Specific Aim 1 - Analyte/Panel Development - *Develop bioluminescent assays for custom panels of specific clinical interest and relevance, including those appropriate to the management of several chronic metabolic diseases.*

Expanding Microscale Bioluminescence to Other Analytes

Calibration and validation studies will be performed for bioluminesce detection of phenylalanine, glucose, glucose 6-phosphate, galactose-1-phosphate (G-1-P), lactose, pyruvate, creatine, and creatinine using the detector design developed in R21. Each assay will be optimized for the physiologic/pathologic concentration range needed for blood and/or urine samples. We will use these analytes to develop 2 example clinical chemistry panels – a renal function panel and a galactosemia panel.

Kidney/Hemodialysis Panel:

Currently the renal panel consists of measuring Albumin, Ca^{++}, CO_2, Cl^-, Creatine, glucose, potassium, sodium, and Blood Urine Nitrate (BUN). We will implement the bioluminescent assays for glucose and creatine. The electrolytes cannot be tested by bioluminescence, but they can easily be measured by electrochemical means that can be incorporated into the same fluidic device. We will implement the electrochemical and bioluminescent sensors on the same device to measure Na^+, and Cl^- in order to show the ability of integrating bioluminescent detection with other methods on the low volume device. Albumin and BUN will not be included on our device at this stage and will be reserved for future research.

The preliminary assessment of the feasibility for direct monitoring by patients of the status of their renal transplant status, was brought to our attention by Dr. John Holman, a renal transplant surgeon at the University of Utah with whom we now have an active collaboration. Through this collaboration we learned that an ideal renal function panel should also include urea and uric acid analyses. There are enzyme-based reactions for these analytes, which can be coupled to ATP-luciferase reactions, thereby providing bioluminescent assays for urea and uric acid. We will also perform calibration studies for these analytes.

The optimal conditions for each bioluminescent analytical assay will be determined by modeling and simulating the multiple reactions using enzyme kinetics modeling software (GEPASI – see www.gepasi.org). This method was applied to creatinine in order to determine its feasibility in bioluminescence detection. Creatinine is readily converted to creatine by a creatinase enzyme and then creatine is phosphorylated with creatine kinase to creatine phosphate. The firefly luciferase sensor detects the consumption of ATP via the phosphorylation reaction. A set of optimum conditions was estimated from the simulation and then preliminary sets of experiments were conducted [16]. The general conclusion from the creatinine experiments and the model was that such a sensor is indeed feasible and merits development. This modeling method can be applied for determining the feasibility for measuring other analytes by bioluminescence.

Hemo- and peritoneal dialysis patients are often chronically malnourished, due in part to dialysis-induced depletion of their circulating amino acid levels. Thus a comprehensive panel for dialysis patients should include some measures of amino acid nutrition, such as phenylalanine. This will be implemented and tested in the luminescence-based microdevice [9].

Galactosemia Panel

Galactosemia is a most interesting metabolic disease. The high circulating galactose concentrations drive several enzyme biochemical reactions, which are normally not important: the production of galactitol and galactonate. Recent clinical research suggests that galactitol in particular is the major "toxin"in galactosemia; its plasma and urine concentrations are likely to be better measures of the severity of galactosemia – and of the dietary management of the condition--than the traditional measures of galactose and galactose-1-phosphate. We will have implemented galactose in the microdevice during the R21 phase and will implement and test galactose-1-phosphate (G-1-P) [13] during this phase. Dr. Chris. Eu from our lab completed an extensive modeling and simulation of the reactions appropriate to the measurement of the metabolites relevant to galactosemia [54].

Buffered solution standards for each analyte will be used for this specific aim.

Specific Aim 2 - Blood and Urine Samples – *Test and validate luminescent analysis with standard urine and plasma samples.*

Urine tests

A creatinine channel is needed for ALL urine analyses. As urine dilution is generally accounted for via the measurement of creatinine, analyte "concentrations" in urine are generally referenced to urine creatinine concentrations. The renal function panel will be tested with commercially available urine samples. Internal standards, known amounts of each analyte will be added for calibration purposes.

Blood Sample

Some analytes are measured in "blood" and some in plasma, or even serum. The major difference between blood and plasma is the red blood cell component. We will incorporate a plasma separation membrane into the analytical microdevice, separating the red cells from the plasma and permitting direct plasma measurements. To perform a blood measurement, an intact blood sample is hemolyzed and the hemolysate plus plasma is measured. If the red blood cell analyte concentration is desired, then the plasma value is subtracted from the total blood value. This, of course, means that the sample hematocrit must be known in order to do the simple calculation.

Plasma separation will be implemented in the microdevice using commercial plasma separation membranes on the sample inlet of the device. Plasma separation membranes are available from several suppliers to the invitro diagnostics (IVD) industry. The membrane permits the passage of plasma and retains the cells. We will evaluate some blood separation membranes, although some suitable for prototype construction and evaluation are already available. For plasma analyses, the membranes should produce minimal hemolysis. We will experiment with surfactant and polyethylene oxide (PEO) copolymer pretreatments, if needed, to minimize hemolysis.

We must also facilitate total blood analysis, by incorporating hemolysis agents (in the absence of a plasma separation membrane) on the inlet channel of the appropriate section of the device. Blood samples may be hemolyzed and the entire solution component analyzed (hemolysate plus plasma); or the red cells may be filtered and only the remaining plasma allowed into the microdevice, providing a plasma analysis. For whole blood measurements the red blood cells are

fully hemolyzed (lysed) and the combined plasma and hemolysate is then processed through the plasma separation membrane, which retains red cell ghosts and membrane fragments. We will investigate several of the common hemolyzing agents already in use in clinical chemistry test kits and protocols. The optimum agents will be further studied with respect to deposition on the microdevices in the sample placement and pre-processing regions of the devices.

A small volume of the whole blood sample will be wicked to a separate channel/section of the microdevice for the measurement of hematocrit (red cell volume); this is best done by measuring the absorbance of the fully oxygenated hemoglobin in the red cell fraction. The initial approach to study this will use an ATP-luciferase bioluminescence standard as the light source, which is then attenuated by the hemoglobin absorbance. By optimization of blood volume, path length, etc. we feel a reasonable hematocrit measurement can be obtained. Should the ATP-luciferase bioluminescence standard not work efficiently, a micro-LED (like the ones used for the detector calibrations) will be used as the light source.

Commercially available blood certified free of HIV and hepatitis antigens will be used. Internal standards, known amounts of each analyte, will be added for calibration purposes to the blood samples. The accuracy, linearity and precision of the assays will be determined. Our results will be referenced against standard clinical chemistry tests. Clinical chemistry services of ARUP, Inc. in Salt Lake City, UT and the Children's Hospital Clinical Labs, Los Angeles, CA, will be used for confirmation of testing results. We will utilize common methods of evaluating analytical performance. These studies will use standard assays and methods of analysis, including receiver operating characteristic (ROC) plots and the Clarke Error Grid analysis commonly used to evaluate glucose analysis strips.

Specific Aim 3 - Quality Assurance - *Minimize measurement errors and develop quality assurance features such as bubble/incomplete well filling detection, onboard calibration channels, and optimum or dual ranging detection.*

This specific aim will focus on minimizing measurement errors associated with chemistry and stabilization, sample preparation and delivery, and device design.

Improved Chemistry

The NADH-dependent oxidoreductase/bacterial luciferase reaction requires an alkyl aldehyde reactant. Although our present assay formulations work well, the aldehyde has some volatility and odor. We propose to produce a hydrophilic, low vapor pressure alkyl aldehyde by reaction with polyethylene glycol (PEG). We will use reactive monofunctional polyethylene glycols (PEG) of 3400 molecular weight. These are available from Shearwater Polymers (www.swpolymers.com) with epoxy, tresylate, or aldehyde functional groups. As these reactive groups require a nucleophilic amine group to couple, the alkyl aldehyde precursor ideally should have a terminal amino group. Fortunately, a 12-amino dodecanoic acid is commercially available (Aldrich/Fluka). This 12 carbon alkyl compound will couple to the reactive PEG. The product, a PEG-alkyl alcohol, will then be oxidized to the aldehyde. The result is an alkyl aldehyde with a large, flexible, hydrophilic PEG chain. This material should function in the bioluminescence reaction, while exhibiting low volatility. In fact, it is likely that this aldehyde will have significantly greater activity that the conventionally used form, due to increased solubility and availability. A highly efficient, enhanced NADH bioluminescent reaction will permit even more sensitive assays for NADH-related analytes, including nearly all of the 20 common amino acids, through amino acid dehydrogenase reaction pathways.

We will also develop means to expand analytical ranges for several analytes in order to be able to detect and compare normal and abnormal levels that may be dramatically vary in concentration.

For example, PKU patients need to measure phenylalanine over the 100 to 1000 microMolar range, while normal, non-PKU patients will need accuracy over the 50 to 300 microMolar range. Dual ranging, which in this case requires two independent analyte channels or wells, is accomplished by optimizing reagent concentrations and conditions to the range of interest. These optimizations can be designed by the enzyme kinetic modeling software, GEPASI, described above.

Stabilization Studies

The stabilization studies performed in R21 will be expanded to test the long-term stability of the lyophilized bioluminescent assays. The study will extend up to 15 months (with some experiments running over from the R21 phase) at 3-month intervals. Stabilization test will be performed for lactate, galactose, glucose, and creatine bioluminescent detection assays within the device.

Sample Filling

The results from the sample delivery and mixing studies in the R21 phase (page 23) will determine what type of system would be best for detecting and indicating insufficient sample deposition, blocked channel or incomplete filling/bubbles in the wells. Such systems could include electrode sensors in the channels to indicate when sufficient sample is applied. The optimized detector determined in specific aim 3 of the R21 phase could also work to detect insufficient filling of the wells. If the detector has multiple pixels (3-4) for each well it can detect irregularities in the expected signal. Should one of the pixels give an abnormally low signal compared to the other pixels for that well, it could indicate a bubble in that well.

Onboard Calibration Standards

We will develop ways to include reference solutions within the final device, or "chip", to function as onboard calibration or reference channels. One method we will try is to have two separate sample reservoirs or inlet channels. As a biofluid ample such as blood or urine is applied to one channel on one side of the device, a reference solution that includes known concentrations of all the analytes to be detected is applied to a second inlet channel on the other side of the device. Each side divides the samples into the analytical wells, where the same assays are prepared. The measurements on the biofluid sample side of the chip are referenced to the measurements from the reference side for each analyte for calibration. Any reduction in activity of the assays would occur for both the reference and biofluid measurements because the same assays would have been prepared on both sides of the chip at the same time. This is an approach similar to glucometers, but may require calibrations for each analytical chip or at least for every batch of chips.

Our results will be referenced against standard enzyme spectrofluorometric assays for each of the analytes. The clinical chemistry services of ARUP, Inc. in Salt Lake City, UT and the Children's Hospital Clinical Labs, Los Angeles, CA, will be used.

We will utilize common methods of evaluating analytical performance. These studies will use standard assays and methods of analysis, including receiver operating characteristic (ROC) plots and the Clarke Error Grid analysis.

Specific Aim 4 - Data Analysis and Multi-Parameter Visualization – *Develop analytical algorithms to facilitate data presentation and usefulness; using the onboard reference and calibration channels, a calibration model will be developed for each analyte and for each type of biological sample (blood, plasma, urine). For some analytes, ratios, differences, or other processing of the raw data will be required to produce a useful, interpretable output.*

As it is difficult to deal with many different channels of information, we will also address what is sometimes called the "cockpit problem" – how does a pilot, for example, deal with the many sensors and their outputs in a modern jet cockpit? How does an anesthesiologist, surgeon, or nurse deal with the myriad of monitors and signals in the typical operating room or intensive care suite? In our case, how do physicians, patients, and family members effectively deal with the interactions among perhaps 20 different analytes? Fortunately, advances in data analysis, parameter presentation, and visualization -- coupled with appropriate modeling, simulation, and sensitivity analyses, allow this challenge to be effectively addressed. Clearly we cannot do justice to this specific aim in this 2 year R33 grant, rather we will get started in this direction and then apply for funds with which to continue the work.

We will experiment with a range of multi-parameter visualization tools; particularly multi-axes radar plots (also called spider, radial, or tetra plots) [55, 56, 57] to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

We present one example here as to the potential of simple methods for the visualization of multi-parameter clinical chemistry data (Table 4)[54].

Table 4 shows how 4 key metabolites and the 3 enzyme activities are altered in the three different types of galactosemia. If we had a seven-channel sensor, we could measure all seven analytes and present the data in a radar plot (Figure 14) to enhance the recognizability of the three types of galactosemia. The "trick" to effective multi-parameter data presentation using such radial plots is to position and scale the axes to produce easily recognizable signatures. Note that the enzyme axes are the reciprocal of enzyme activity. Normality is represented by the inner hexagon (made by connecting the points on each of the 6 individual concentration axis). Type II galactosemia "points" to the left, type I to the top, and type III to the right.

Table 4: The concentration (or activity) change of metabolites and enzymes in whole blood for different types of galactosemia.

	Type-I Galactosemia (GALK deficient)	Type-II Galactosemia (GALT deficient)	Type-III Galactosemia (GALE deficient)
GALK	↓↓↓	—	—
GALT	—	↓↓↓[a]	—
GALE	—	—	↓↓↓
Gal[b]	↑↑↑	↑↑	↑↑
Gal-1-P	↑↑	↑↑↑	↑↑
Galactitol[b]	↑↑↑	↑↑↑	N.A.
Galactonate[b]	↑↑↑	↑↑↑	N.A.

↑↑↑ (highly increased), ↑↑ (moderately increased), ↓↓↓ (highly decreased), — (unchanged), N.A. (not available). [a] Variants of Type II galactosemia have higher GALT activity. [b] Elevated concentrations of these metabolites are also found in urine.

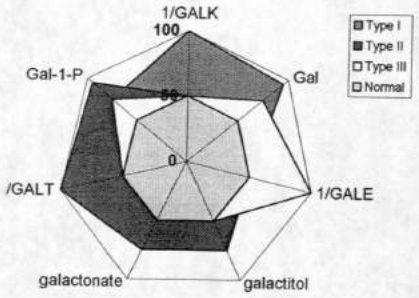


Figure 14: A preliminary Radar Plot for Galactosemia. Normality is represented by the inner locus, the various galactosemia types by the other 3 patterns.

It is important to note that we can design sensing channels for enzyme concentration (activity) as well as for substrate or product activity. If we want to measure a particular "substrates" activity, we produce a channel with an excess of substrate and co-reactants and look for product (via an ATP of NADH coupled reaction). We have already done this for galactokinase activity (Type I) [58]. We have also done similar analyses with multi-amino acid data from PKU patients and are now looking at other data sets from the NHANES national clinical chemistry database.

The proposed μAS will in the end, be able measure up to 100 different metabolites in the sub-microMolar to milliMolar range from one 1-100 μL biofluid sample, and include customized comprehensive diagnostic panels. The device will also provide an initial platform where which clinicians, researchers and even lay patient end users will be able to interpret the complex data that will be presented to them.

E. Human Subjects – None

F. Vertebrate Animals - None

G. Literature Sited

1. A.P.F. Turner, "Biosensors – Sense and Sensitivity" *Science*, 17 Nov. 2000, Vol. 290, 1315-1317.
2. Turner, G.K., "An Introduction to Chemiluminescence and Bioluminescence Measurements," http://www.turnerdesigns.com/t2/doc/appnotes/998_2620.html
3. S. Brolin and G. Wettermark, *Bioluminescence Analysis*, VCH Publ., 1992.
4. Berthold F, Herrick K, Seiwe RM. Luminometer Desing and Low Light Detection. *Methods of Enzymology* 1999;**305**:62-87.
5. Campbell AK. Chemiluminescence. Chichester, England: VCH, 1985.
6. Lundin A. Use of Firefly Luciferase in ATP Assays of Biomass, Enzymes, and Metabolites. *Methods of Enzymology* 2000;**305**:346-370.
7. Fullman PS, Fullman BS. Comparison of Real Life Luminometer Sensitivity as Determined with Actual Luminescence Assays. *Bioluminescence and Chemiluminescence* :553-556.
8. Michael G, ed. Biochemical Pathways. New York: Wiley, 1999.
9. Min D, Stewart R, Andrade J. Biosensor for Phenylalanine Based on Bacterial Bioluminescence. In: Roda Aea, ed. Bioluminescence and Chemiluminescence: Perspectives for the 21st Century: Wiley, 1999: 520-523.
10. Idahl LA, Sandstrom PE, Sehlin J. Measurements of serum glucose using the luciferin/luciferase system and a liquid scintillation spectrometer. *Anal Biochem* 1986;**155**(1):177-81.
11. Wienhausen G, DeLuca M. Bioluminescent assays of picomole levels of various metabolites using immobilized enzymes. *Anal Biochem* 1982;**127**(2):380-8.
12. J. Y. Eu, C. Y. Wang and J. Andrade, "Homogeneous bioluminescence assay for galactosuria: interference and kinetic analysis," *Anal Biochem*, vol. 271(2), pp. 168-76., 1999.
13. Wilson RG, Riordan PR, Hartmann PE. Galactosaemia: sensitive bioluminescent assays for galactose and red cell galactose-1-phosphate. *J Inherit Metab Dis* 1990;**13**(3):270-2.
14. Arthur PG, Kent JC, Hartmann PE. Microanalysis of the metabolic intermediates of lactose synthesis in human milk and plasma using bioluminescent methods. *Anal Biochem* 1989;**176**(2):449-56.
15. Min D. Ph.D. Dissertation. University of Utah, 1999.
16. Davies R, Eu J, Min D, Andrade JD. Bioluminescence Assays For Creatine And Creatinine For Biosensor Applications. Biosensors Conference 2000 2000, San Diego: 319.
17. Thompson MJ, Arthur PG, Hartmann PE. Bioluminescent methods for determining metabolites in micro-samples of pig plasma. *Eur J Clin Chem Clin Biochem* 1997;**35**(11):861-6.
18. T. H. Grove, "In Vitro Diagnostics: Bringing Testing to the Point of Care," *Medical Device & Diagnostics Industry*, 78-94, April 2000.
19. Min DJ, Andrade JD, Stewart RJ. Specific immobilization of in vivo biotinylated bacterial luciferase and FMN:NAD(P)H oxidoreductase. *Anal Biochem* 1999; 270(1):133-9.

20. Davies R, Van Wagenen RA, Andrade J. Stabilization of firefly luciferase with anti-oxidants: a preliminary study. 11th International Symposium on Bioluminescence and Chemiluminescence 2000, Monterey, California.
21. Wang CY, Hitz S, Andrade JD, Stewart RJ. Specific immobilization of firefly luciferase through a biotin carboxyl carrier protein domain. *Anal Biochem* 1997;**246**(1):133-9.
22. Kim YD, Park CB, Clark DS. Stable sol-gel microstructured and microfluidic networks for protein patterning. *Biotechnology and Engineering* 2001;**73**(5):331-337.
23. D.C. Duffy, J. McDonald, O. Schueller, G. Whitesides, "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)," *Anal. Chem.*, vol. 70, no. 23, pp. 4974-4984, December 1, 1998.
24. J. R. Anderson, D. T. Chiu, R. J. Jackman, O. Cherniavskaya, J. C. McDonald, H. Wu, S. H. Whitesides and G. M. Whitesides, "Fabrication of topologically complex three-dimensional microfluidic systems in PDMS by rapid prototyping," *Anal Chem*, vol. 72(14), pp. 3158-64., 2000.
25. Y. Xia and G. M. Whitesides, "Soft Lithography," *Annu. Rev. Mater. Sci.*, vol. 28,pp. 153-184, 1998.
26. R. J. Jackman, D. C. Duffy, E. Ostuni, N. D. Wilmore and G. M. Whitesides, "Fabricating Large Arrays of Microwells with Arbitrary Dimentions and Filling Them Using Discontinuous Dewetting," *Anal. Chem.*, vol. 70(11), pp. 2280-2287, 1998.
27. A. L. Hart, A. P. F. Turner and D. Hopcroft, "On the Use of Screen and Ink-jet Printing to Produce Ampermetric Enzyme electrodes for Lactate," *Biosensors & Bioelectronics*, vol. 11(3), pp. 263-270, 1996.
28. Y. D. Kim, C. B. Park and D. S. Clark, "Stable sol-gel microstructured and microfluidic networks for protein patterning," *Biotechnology and Engineering*, vol. 73(5), pp. 331-337., 2001.
29. F. Arias, S. R. J. Oliver, B. Xu, R. E. Holmlin and G. M. Whitesides, "Fabrication of Metallic Heat Exchangers Using Sacrificial Polymer Mandrils," *Journal Of Microelectromechanical Systems*, vol. 10(1), pp. 107-112, 2001.
30. A. Rasmussen, M. Gaitan, L. E. Locascio and M. E. Zaghloul, "Fabrication Techniques to Realize CMOS-Compatible Microfluidics in Microchannels," *Journal of Microelectromechanical Systems*, vol. 10(2), pp. 286-267, 2001.
31. G. T. A. Kovaks, *Micromachined Transducers Sourcebook*, 1, WCB McGraw-Hill, 1998.
32. Kurt Petersen, William McMillan, Gregory Kovacs, Allen Northrup, Lee Christel, and Farzad Pourahmadi, "The Promise Of Miniaturized Clinical Diagnostic Systems," *IVD Technology Magazine*, July, 1998.
33. Sainato D. Do Hospitals Draw Too Much Blood? Clinical Laboratory News, 1999: 1.
34. J. D. Brazzle, D. A. Bartholomeusz, R. Davis, J. D. Andrade, R. VanWagenen, A. B. Frazier, "Active Microneedles with Integrated Functionality," *Solid State Sensors and Actuators Conference*, Transducers Research Foundation, June 4-8, 2000.
35. D. A. Bartholomeusz, J. D. Andrade and A. B. Frazier, "Bioluminescent based chemchip for point-of-care diagnostics," in *1st Annual International, Conference On*

Microtechnologies in Medicine and Biology, Palais des Congres, Lyon, France, October 12-14, 2000.
36. Internal research from a former start-up company (*Protein Solution, Inc.*) associated with our research group.
37. Maresch M., "The Linear CCD array", Sun Jun 15 21:47:32 MDT 1997 http://www.icg.tu-graz.ac.at/ORGroup/Staff/maresch/pub/thesis/node30.html
38. Grove TH. In Vitro Diagnostics: Bringing Testing to the Point of Care. Medical Device & Diagnostic Industry, 2000: 78-94.
39. I. Levine, *Physical Chemistry 4th ed.*, McGraw-Hill, 1995, pg. 685.
40. R. Davies, R. A. Van Wagenen and J. Andrade, "Stabilization of firefly luciferase with anti-oxidants: a preliminary study," in *11th International Symposium on Bioluminescence and Chemiluminescence*, Monterey, California, 2000.
41. Kurt Petersen, William McMillan, Gregory Kovacs, Allen Northrup, Lee Christel, and Farzad Pourahmadi, "The Promise Of Miniaturized Clinical Diagnostic Systems" *IVD Technology Magazine*, July, 1998.
42. D. J. Kane and J. Lee, "Absolute Calibration of Luminometers with Low-Level Light Standards", in *Bioluminescence and Chemiluminescence*, Methods of Enzymology, M. M. Ziegler and T. O. Baldwin Eds., vol. 305 Part C, Academic Press, pp. 87-96, 2000.
43. BioThema, The Firefly Luciferase Reaction: Applications, Optimization and Standardization. Bioluminescence and Chelilumínescence 2000: 61.
44. Mizoguchi N, Ono H, Eguchi T, Sakura N. Galactose metabolites in blood from neonates with and without hypergalactosaemia detected by mass screening. *Eur J Pediatr* 2000;**159**(11):851-3.
45. Grove TH. In Vitro Diagnostics: Bringing Testing to the Point of Care. Medical Device & Diagnostic Industry, 2000: 78-94.
46. Despont M, Lorenz H, Fahrni N, Brugger J, Renaud P, Vettiger P. High-aspect-ratio, ultrathick, negative-tone near-uv photoresist for MEMS applications. IEEE Micro Electro Mechanical SystemsTenth Annual International Workshop 1997: 518 -522.
47. Kobayashi; Hironori, Yamamoto; Manabu, Aoki; Daigo, Kamiyama; Hironori, Hikosaka; Shinichi, Kashiwabara; Mitsuhiro, United States Patent 6,294,313, February 18, 1999.
48. Arias F, Oliver SRJ, Xu B, Holmlin RE, Whitesides GM. Fabrication of Metallic Heat Exchangers Using Sacrificial Polymer Mandrils. *Journal Of Microelectromechanical Systems* 2001;**10**(1):107-112.
49. B. H. Jo, L. M. Van Lerberghe, K. M. Motsegood and D. J. Beebe, "Three-Dimensional Micro-Channel Fabrication in Polydimethylsiloxane (PDMS) Elastomer," *Journal Of Microelectromechanical Systems*, vol. 9(1 (mar)), pp. 76-81, 2000.
50. Y. Lee, I. Jablonski and M. DeLuca, "Immobilization of firefly luciferase on glass rods: properties of the immobilized enzyme," *Anal Biochem*, vol. 80(2), pp. 496-501, 1977.
51. C. Y. Wang, S. Hitz, J. D. Andrade and R. J. Stewart, "Specific immobilization of firefly luciferase through a biotin carboxyl carrier protein domain," *Anal Biochem*, vol. 246(1), pp. 133-9, 1997.

52. G. Wienhausen and M. DeLuca, "Bioluminescent assays of picomole levels of various metabolites using immobilized enzymes," *Anal Biochem*, vol. 127(2), pp. 380-8, 1982.
53. Bland JM, Altman DG. Satistical Methods for Assessing Agreement Between Two Methods of Clinical Measurement. *The Lancet* 1986;Feb:307-310.
54. Eu J. Thesis. Ph.D.: University of Utah, 2000.
55. Cerra FB. Hypermetabolism, organ failure, and metabolic support. *Surgery* 1987;**101**(1):1-14.
56. Andrade J. Proteins at Interfaces. *Clinical Materials* 1992;**11**:67-84.
57. Vogt W, Nagel D, Sator H. Cluster Analysis in Clinical Chemistry. Chichester: John Wiley & Sons, 1987.
58. Eu J, Andrade J. Bioluminescence Assay for Galactokinase Activity. In: Roda Aea, ed. Bioluminescence and Chemiluminescence: Perspectives for the 21st Century: Wiley, 1999: 489-492.

H. Consortium/Contractual Agreements – None

I. Consultants – None

3 Annual Progress Reports →

Form Approved Through 5/2004

OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Progress Report

Review Group	Type	Activity	Grant Number
			1 R21 RR17329-01

Total Project Period: From: 8/1/02 Through: 7/31/06

Requested Budget Period: From: 8/1/03 Through: 7/31/04

1. TITLE OF PROJECT
Multi-Analyte Micro-Devices for Biomedical Applications

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Name and address, street, city, state, zip code)
Joseph D. Andrade, Ph.D.
50 S. Central Campus Dr.
2480 MEB
Salt Lake City, UT 84112-9202

3. APPLICANT ORGANIZATION (Name and address, street, city, state, zip code)
University of Utah
1471 Federal Way
Salt Lake City, UT 84102

2b. E-MAIL ADDRESS
joeandrade@uofu.net

4. ENTITY IDENTIFICATION NUMBER
1876000525A1

2c. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Bioengineering

2d. MAJOR SUBDIVISION

5. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL
Vincent Bogdanski: Manager, Grants and Contracts
1471 FEDERAL WAY
SALT LAKE CITY UT 84102-9020

E-MAIL: ospawards@osp.utah.edu

6. HUMAN SUBJECTS
☒ No ☐ Yes
6a. Research Exempt ☐ No ☐ Yes
6b. Human Subjects Assurance No.
If Exempt ("Yes" in 6a): Exemption No.
6c. NIH-Defined Phase III Clinical Trial ☒ No ☐ Yes
If Not Exempt ("No" in 6a): IRB approval date
☐ Full IRB or ☐ Expedited Review

7. VERTEBRATE ANIMALS
☒ No ☐ Yes
7a. If "Yes," IACUC approval Date
7b. Animal Welfare Assurance No.

8. COSTS REQUESTED FOR NEXT BUDGET PERIOD
8a. DIRECT $
8b. TOTAL $

9. INVENTIONS AND PATENTS
☒ No ☐ Yes If "Yes," ☐ Previously Reported ☐ Not Previously Reported

10. PERFORMANCE SITE(S) (Organizations and addresses)
University of Utah
50 S Campus Center Dr
2405 MEB
Salt Lake City UT 84112-9202

11a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Item 2a)
Joseph D. Andrade, Ph.D.
TEL 801 581-4379
FAX 801 585-5361

11b. ADMINISTRATIVE OFFICIAL NAME (Item 5)
Vincent A. Bogdanski
TEL 801 581-3008
FAX 801 581-3007

11c. NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Item 14)
NAME Vincent Bogdanski
TITLE Acting Co-Director, Office of Sponsored Projects
TEL 801 581-3003 FAX 801 581-3007
E-MAIL ospawards@osp.utah.edu

12. Corrections to Page 1 Face Page
There are no changes to the direct and total costs requested for the next budget period.

13. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.
SIGNATURE OF PI/PD NAMED IN 2a. (In ink. "Per" signature not acceptable.)
DATE 5-29-03

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.
SIGNATURE OF OFFICIAL NAMED IN 11c. (In ink. "Per" signature not acceptable.)
DATE 5/30/03

Principal Investigator/Program Director (Last, first, middle): Andrade, Joseph D.

PROGRESS REPORT SUMMARY

GRANT NUMBER 1 R21 RR17329-01

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	PERIOD COVERED BY THIS REPORT FROM	THROUGH
Joseph D. Andrade, Ph.D.	8/1/02	7/31/03

APPLICANT ORGANIZATION
University of Utah: Department of Bioengineering

TITLE OF PROJECT (Repeat title shown in Item 1 on first page)
Multi-Analyte Micro-Devices for Biomedical Applications

A. Human Subjects (Complete Item 6 on the Face Page)
Involvement of Human Subjects ☒ No Change Since Previous Submission ☐ Change

B. Vertebrate Animals (Complete Item 7 on the Face Page)
Use of Vertebrate Animals ☒ No Change Since Previous Submission ☐ Change

SEE PHS 2590 INSTRUCTIONS.

WOMEN AND MINORITY INCLUSION: See PHS 398 Instructions. Use Inclusion Enrollment Report Format Page and Targeted/Planned Enrollment Format Page.

1) There is no change in the other support of key personnel since the last reporting period.
2) There will not be, in the next budget period, a significant change in the level of effort for key personnel from what was approved for this project.
3) It is not anticipated that an estimated unobligated balance (including prior year carryover) will be greater than 25 percent of the current year's total budget.

A. SPECIFIC AIMS

There are no changes to Specific Aim 1 and Specific Aim 2 as stated in the original, competing application. They remain as follows:

SPECIFIC AIM 1: *Detection Limits and Scale Optimization* - Design microscale chambers of various shapes and sizes and determine detection limits for specific bioluminescent analytical assays.
SPECIFIC AIM 2: *Reagent Packaging and Sample Delivery* - Engineer methods for packaging dry reagents in microfabricated chambers. Analyze transport, mixing, and diffusion characteristics of sample liquids with the dried reagents.

Specific Aim 3 in the original proposal, was slightly changed before the final grant was awarded. The changes were based on recommendations from the Scientific Review Group and personal conversation with Dr. Gregory Farber as directed in a letter from Dr. Michael T. Marron in the Summary Statement of the Scientific Review dated April 6, 2002. Instead of building custom photodiode arrays, we are using commercially available photodetector arrays. Additional time and resources saved by the availability of detector arrays are being spent on addressing biosensor fabrication and microfluidic issues of the other specific aims. Specific Aim 3 is:

SPECIFIC AIM 3: *Detector Selection* - Configure and compare various commercially available photodetector arrays and their detection limits when applied to the bioluminescence based sensor. The milestones for Specific Aim 3 remain unchanged from the final milestones of the awarded grant.

There were no budget modifications by the Scientific Review Group and Council.

B. STUDIES AND RESULTS

SPECIFIC AIM 1: *Instrument Calibration*: We develop a method for calibrating and comparing photodetectors in standardized units of (photons/sec/steradian) using a light emitting diode (LED) as a light source standard. This method can be used for calibrating bioluminescence and chemiluminescence based analytical assays and determining their detection limits. The method is described in section E (Publications).

Detection Limits: We have achieved the detection limits, as specified in Milestones 1 and 2, for measuring 2 proposed analytes (ATP and NADH) using lyophilized bioluminescence based assays (See Table 1pg 4). We still need to perform detection limit experiments for galactose and lactate bioluminescence-based assays.

Biosensor Fabrication: We have built 5×5 arrays of sub-microliter wells in PolyDimethylsiloxane (PDMS) and bonded them to glass as outlined in the proposal (Figure 1 pg 4). However, we have not been able to achieve better than a 50% yield in fabrication. The wells deform, making precise volume control difficult. This is in part due to limited capabilities of the functional plasma etcher in the University of Utah microfabrication facility (the facility is now under major renovation and is difficult to access some equipment). Because of this, we have looked into other prototyping methods and materials and found successful alternatives.

The most promising prototyping method we investigated was to laser cut holes in opaque plastic and cover the bottom of the holes with a clear membrane coated with an adhesive. We are currently testing white and black High Density Polyethylene (HDPE) and Polystyrene. Clear, medical grade adhesive polyester films (available from Adhesives Research, Inc.) were bonded to polyethylene and polystyrene substrates with holes and bioluminescent reagents were added to the wells. The adhesive films survived the lyophilization process without any of the reagents leaking or the film pealing away from the substrates. Further tests with these plastic chips are outlined in section D (Plans).

Light Collection Enhancements: We will address light collection enhancement features once the basic versions of the plastic prototype (mentioned above) are tested.

SPECIFIC AIM 2: *Lyophilization and Stabilization*: We have been able to stabilize reagents within an array of PDMS wells bonded to glass. Currently, these wells are filled by manually pipetting the reagents into the wells. In order to accurately test reagent and sample volume less than 0.4 µL, we have built a computer controlled syringe pump/solenoid micro dispenser (Figure 2 pg 4). The variability in the drops is still too great (greater than 40%) due to an unstable pump. We are currently addressing this issue by with a pressure regulator.

Sample Delivery & Mixing: Limited results. The lyophilized reagents rapidly rehydrate when sample fluids are added to the top of the array. We have achieved CCD measurements of the luminescence occurring in each well when a hydrogel saturated with a sample fluid is placed on top of the array (Figure 3 pg 4).

SPECIFIC AIM 3: *Detector Comparison*: We have purchased photodetector arrays as well as the analog to digital equipment to control and read the luminescent signals. We will begin assembling these items during the next budget year.

Supplements: Not Applicable.

C. SIGNIFICANCE

The ability to measure ATP and NADH, at the detection levels specified in Table 1 (pg 4), and in a stabilized form, suggests that multiple analytes (coupled to the ATP and NADH bioluminescent reactions) can be measured from small sample volumes in a new type of Micro-Analytical System (µAS) that can be operated at point-of-care and home environments. It will likely evolve to measure up to 100 different metabolites in the sub-microMolar to milliMolar range from one 1-100 µL biofluid sample, and include customized comprehensive diagnostic panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs.

D. PLANS

SPECIFIC AIM 1: We will determine the detection limits and ranges for bioluminescence-based measurement of lactate and galactose, as well as ATP and NADH. Measurements will be done with photomultiplier tube and CCD detectors using the biosensors fabricated in HDPE and Polystyrene as described above.

SPECIFIC AIM 2: In addition to the microfluidic sample delivery methods outlined in the proposal, we will address sample delivery and rehydration by testing porous flow through membranes on top of the array of holes as a method of sample delivery (Figure 4 pg 4). This method is based on the rapid rehydration success we had with the hydrogels that were soaked in a sample fluid (Figure 3 pg 4).

SPECIFIC AIM 3: We will assemble and test the photodetector arrays and determined the detection limit of each of the proposed analytes with each detector.

E. PUBLICATIONS

1. Bartholomeusz, D.A. and Andrade, J.D., "Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units" in *Bioluminescence and Chemiluminescence: Progress and Current Applications*. 2002. Robinson College, University of Cambridge, UK: World Scientific. p. 189-192. Abstract in *Luminescence: Journal of Biological and Chemical Luminescence*, 2002. 17(2): p. 80.
2. Davies, R., Bartholomeusz, D.A., and Andrade, J., "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" in *IEEE Engineering in Medicine and Biology Magazine*, 2003. **22**(1): p. 32-42.
3. Davies, R.H., Andrade, J.D. "Diabetes-Related Luminescent Assays for Multi Analyte Measurement" in *Diabetes Technology & Therapeutics*, 2003, **5**(2): p. 237-238.

F. PROJECT-GENERATED RESOURCES

The LED calibration method described in section B can be beneficial for standardizing photodetector measurement units for accurate detector comparison. The procedure is outlined in the list of publications and will be prepared for a peer reviewed journal.

Tables and Figures

Table 1: Detection limits Achieved for ATP and NADH in Lyophilized Bioluminescence-Based Assays

Analyte	Minimum Sample Concentration	Sample Volume	Experimental Detection Limit (lyophilized form) (error less than ± 20%)	Detection Limit required for Milestone 1 (wet assays) (error less than ± 10%)	Detection Limit required for Milestone 2 (lyophilized assays) (error less than ± 20%)	Time of Assay
ATP	1 µM	0.5 µL	0.5 picomoles	10 picomoles	100 picomoles	30 seconds
NADH	50 µM	1.0 µL	313 picomoles	200 picomoles	2,000 picomoles	3 minutes

The above experimental detection limits for ATP and NADH bioluminescence based assays were done in lyophilized form. These detection limits meet the Milestone 2 requirements for these analytes. The wet *bioluminescence based assays for these analytes (not shown) had 10x better detection limits, satisfying* Milestone 1 requirements for these analytes. Lactate and Galactose detection limits still need to be performed.

Figure 1: Array of Wells in PDMS

A 5×5 array of 0.4 µL wells molded in PDMS and bonded to a glass cover slip. (reagents not added in this picture).

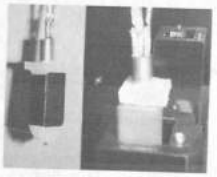


Figure 2: Micro-dispenser in Spectrophotometer

A spectrophotometer was used to calculate drop volumes. drop volumes of 40 ± 30 nL for the 0.0050" ID nozzle and 200 ± 80 nL for the 0.0075" ID nozzles The miniature air pump (not shown) did not function as a constant pressure source, increasing the error of the measured drop volumes. Since these calibrations have been performed, we have stabilized the pressure source and are recalibrating the dispenser *with nozzles 0.0050" ID and smaller.*

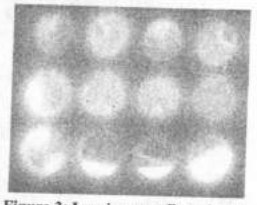
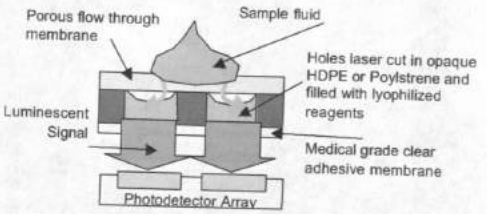


Figure 3: Luminescent Reaction in an Array of Wells in PDMS

120 sec CCD exposure of luminescence in 0.4 µL wells from 1 µM ATP solution mixing and reacting with a firefly luciferase platform that has been lyophilized and stored for 20 days.

Figure 4 (right): Through Flow Membrane Facilitating Sample Delivery into Lyophilized Reagents

We tested medical grade adhesive membranes on HDPE and polystyrene substrates with holes filled with bioluminescent reagents. The adhesive films survived the lyophilization process without any of the reagents leaking or the film pealing away from the substrates. During the next budget period, we will test microfluidic sample delivery including sample delivery through porous through flow membranes bonded to the top of the HDPE and polystyrene substrates. These basic prototypes will also be used to test the commercial photodetector arrays. These photodetector arrays can be used in a hand held point-of-care device.



Form Approved Through 5/2004 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Progress Report

Review Group	Type	Activity	Grant Number
			1 R21 RR17329-02

Total Project Period From: 8/1/02 Through: 7/31/06

Requested Budget Period: From: 8/1/04 Through: 7/31/05

1. TITLE OF PROJECT
Multi-Analyte Micro-Devices for Biomedical Applications

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Name and address, street, city, state, zip code)
Joseph D. Andrade, Ph.D.
50 S. Central Campus Dr.
2480 MEB
Salt Lake City, UT 84112-9202

3. APPLICANT ORGANIZATION (Name and address, street, city, state, zip code)
University of Utah
1471 Federal Way
Salt Lake City, UT 84102

2b. E-MAIL ADDRESS
joeandrade@uofu.net

4. ENTITY IDENTIFICATION NUMBER
1876000525A1

2c. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Bioengineering

5. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL
Vincent A. "Bo" Bogdanski, Assistant Director
Office of Sponsored Projects
1471 FEDERAL WAY
SALT LAKE CITY UT 84102-9020
E-MAIL: ospawards@osp.utah.edu

2d. MAJOR SUBDIVISION
College of Engineering

6. HUMAN SUBJECTS
☒ No ☐ Yes
6a. Research Exempt ☐ No ☐ Yes
6b. Human Subjects Assurance No.
If Exempt ("Yes" in 6a): Exemption No.
6c. NIH-Defined Phase III Clinical Trial ☒ No ☐ Yes
If Not Exempt ("No" in 6a): IRB approval date
☐ Full IRB or ☐ Expedited Review

7. VERTEBRATE ANIMALS
☒ No ☐ Yes
7a. If "Yes," IACUC approval Date
7b. Animal Welfare Assurance No.

8. COSTS REQUESTED FOR NEXT BUDGET PERIOD
8a. DIRECT $ 193,400
8b. TOTAL $ 289,133

9. INVENTIONS AND PATENTS
☒ No ☐ Yes If "Yes," ☐ Previously Reported ☐ Not Previously Reported

10. PERFORMANCE SITE(S) (Organizations and addresses)
University of Utah
50 S Campus Center Dr
2405 MEB
Salt Lake City UT 84112-9202

11a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Item 2a)
Joseph D. Andrade, Ph.D.
TEL 801 581-4379
FAX 801 585-5361

11b. ADMINISTRATIVE OFFICIAL NAME (Item 5)
Vincent A. "Bo" Bogdanski
TEL 801 581-3008
FAX 801 581-3007

11c. NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Item 14)
NAME Elliott Kulakowski, Director
TITLE Office of Sponsored Projects
TEL 801 581-6903
FAX 801 581-3007
E-MAIL ospawards@osp.utah.edu

12. Corrections to Page 1 Face Page
There are no changes to the direct and total costs requested for the next budget period.

13. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.
SIGNATURE OF PD/PI NAMED IN 2a (In ink. "Per" signature not acceptable)
DATE 05/27/2004

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.
SIGNATURE OF OFFICIAL NAMED IN 13 (In ink. "Per" signature not acceptable)
Per V. Bogdanski Signing for
DATE 5-27-04

List of Publications

1. Bartholomeusz, D.A. and Andrade, J.D., "Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units" *Bioluminescence and Chemiluminescence: Progress and Current Applications*. 2002. Robinson College, University of Cambridge, UK: World Scientific. p. 189-192. Abstract in *Luminescence: Journal of Biological and Chemical Luminescence*, 2002. 17(2): p. 80.
   Copy of Published Abstract ..... Publication Appendix pg. 2
   Copy of Published Article..... Publication Appendix pg. 3-6
   Copy of the Presented Poster..... Publication Appendix pg. 7

2. Davies, R., Bartholomeusz, D.A., and Andrade, J., "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" in *IEEE Engineering in Medicine and Biology Magazine*, 2003. **22**(1): p. 32-42.
   Copy of Published Article..... Publication Appendix pg. 8-18

3. Davies, R.H., Andrade, J.D. "Diabetes-Related Luminescent Assays for Multi Analyte Measurement" Abstract in *Diabetes Technology & Therapeutics*, 2003, **5**(2): p. 237-238.
   Copy of Published Abstract ..... Publication Appendix pg. 19-20
   Copy of the presented poster ..... Publication Appendix pg. 21

4. Davies, R.H., Corry, J., Abernathy, J., Andrade, J.D. "Enzymes lyophilized as part of a small-volume bioluminescent multi-analyte quantitative detection system" presented in the 2002 Colorado Protein Stability Conference, Breckenridge, Colorado. There are no published conference proceedings.
   Copy of the presented poster ..... Publication Appendix pg. 22

Principal Investigator/Program Director (Last, first, middle): Andrade, Joseph D.

PROGRESS REPORT SUMMARY

GRANT NUMBER
1 R21 RR 17329-02

PERIOD COVERED BY THIS REPORT

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	FROM	THROUGH
Joseph D. Andrade, Ph.D.	8/1/03	7/31/04

APPLICANT ORGANIZATION
University of Utah: Department of Bioengineering

TITLE OF PROJECT (Repeat title shown in Item 1 on first page)
Multi-Analyte Micro-Devices for Biomedical Applications

A. Human Subjects (Complete Item 6 on the Face Page)
Involvement of Human Subjects ☒ No Change Since Previous Submission ☐ Change

B. Vertebrate Animals (Complete Item 7 on the Face Page)
Use of Vertebrate Animals ☒ No Change Since Previous Submission ☐ Change

SEE PHS 2590 INSTRUCTIONS.
WOMEN AND MINORITY INCLUSION: See PHS 398 Instructions. Use Inclusion Enrollment Report Format Page and Targeted/Planned Enrollment Format Page.

1) There is no change in the other support of key personnel since the last reporting period.
2) There will not be, in the next budget period, a significant change in the level of effort for key personnel from what was approved for this project. There are no additional Key Personnel involved.
3) It is not anticipated that an estimated unobligated balance (including prior year carryover) will be greater than 25 percent of the current year's total budget (indeed, it will be about 0%).

MILESTONES for R21 (from Notice of Grant Award):

1) A microdevice with appropriate reservoirs will be fabricated using PDMS. This device will allow the concentration of ATP, NADH, galactose, and lactate to be determined using bioluminescent based chemical assays. A CCD, or another detector array, will be used as the detector for these assays. For ATP, the device must be able to detect 10 picomoles with a maximum error of 1 picomole. For NADH, the device must be able to detect 200 picomoles with a maximum error of 20 picomoles. For galactose, the device must be able to detect 20 picomoles in a 1 microliter volume with a maximum error of 2 picomoles. The device must be able to determine galactose concentrations in the range from 20 to 90 micromolar. For lactate, the device must be able to detect 500 picomoles in a 1 microliter volume with a maximum error of 50 picomoles. The device must be able to determine lactate concentrations in the range from 0.5 to 2.5 millimolar;

2) Using the microdevice fabricated in milestone 1, methods will be developed to lyophilize the assay mixture in the microdevice. At the conclusion of this milestone, the following detection limits should be achievable using a lyophilized assay mixture. For ATP, the device must be able to detect 100 picomoles with a maximum error of 20 picomoles. For NADH, the device must be able to detect 2 nanomoles with a maximum error of 400 picomoles. For galactose, the device must be able to detect 200 picomoles in a 1 microliter volume with a maximum error of 40 picomoles. The device must be able to determine galactose concentrations in the range from 200 to 900 micromolar. For lactate, the device must be able to detect 5 nanomoles in a 1 microliter volume with a maximum error of 1 nanomole. The device must be able to determine lactate concentrations in the range from 5 to 25 millimolar. The sample fluid for these assays must flow from an input port and fill each well without any technical difficulty or complicated pumps. The sample fluid must fill greater than 95% of the wells without bubbles. A CCD, or another detector array, will be used as the detector for these assays; and

3) Build a detector array or adapt a commercially available detector array and coupling mechanism for the microdevice fabricated in milestone one. This array will be used to measure the concentrations of ATP, NADH, galactose, and lactate using lyophilized assay mixtures. For ATP, the device must be able to detect 10 picomoles with a maximum error of 2 picomoles. For NADH, the device must be able to detect 200 picomoles with a maximum error of 40 picomoles. For galactose, the device must be able to detect 20 picomoles in a 1 microliter volume with a maximum error of 4 picomoles. The device must be able to determine galactose concentrations in the range from 20 to 90 micromolar. For lactate, the device must be able to detect 500 picomoles in a 1 microliter volume with a maximum error of 1 picomole. The device must be able to determine lactate concentrations in the range from 0.5 to 2.5 millimolar.

MILESTONE RESULTS

1) Microdevices with Wet Reagents:

The ChemChip fabrication was simplified since last year's progress report for cost effectiveness and prototyping flexibility. 15mm squares with a 5x5 array of 1mm diameter holes spaced 2 mm apart, were cut out of 0.180 mm thick adhesive backed vinyl film with a knife plotter used for conventional vinyl sign making. The ChemChip patterns were then sealed to 15mm square glass cover slips after manually removing the cut holes. The glass cover slips became the clear bottom for the 140 nL wells (Figure 1).

The chips were loaded, both manually and via a computer controlled syringe pump/solenoid auto dispensing system (Figure 2), with the needed reagent cocktails. A miniature solenoid valve with a 0.002" nozzle (the Lee Company) was used to dispense the reagents (10 ms pulses, pressurized at 8 PSI). The resultant drops were calibrated at 360±10nL. A computer controlled XYZ stage positioned a tray of 25 ChemChips and dispensed reagent drops into individual wells (Figure 2).

The detection limits for wet ATP and NADH were already met in last year's Progress Report; even lower detection limits were achieved recently using a commercial CCD imaging detector.

2) Microdevices with Lyophilized Reagents:

The detection limits for dry ATP and NADH were already met in last year's Progress Report. We have optimized the lyophilization protocols and applied them to the fabrication of multi-channel ChemChips appropriate to Milestone 2.

The reagents were dispensed as noted above onto a tray of ChemChips cooled to less than –60 degrees C using dry ice (Figure 2 left shows the tray of chips on the cold plate)—this allows the reagent droplets to freeze within seconds of dispensing. This process prevented evaporation and maintained reagent stability prior to lyophilization. Since drops were larger than the volume of the wells, a convex meniscus formed above each well.

Lyophilization was performed in two stages in a VirTis Genesis 12 pilot plant lyophilizer. The chips were placed in the sample chamber of the lyophilizer, which had been previously cooled to at least –50° C. Primary lyophilization began when the sample chamber was connected to the condenser chamber cooled to at least -70° C and the system pressure was below 100 milliTorr. Primary lyophilization was performed for 48-72 hours. After the temperature of the sample chamber is changed to 25 ° C, secondary lyophilization was performed for 12-24 hours.

The lyophilized reagents rapidly rehydrate when sample fluids are added to the top of the array. In other studies dealing with analytes in hydrogel contact lenses, we realized that the ChemChip can be easily filled from the top – vertical delivery. We adopted this approach to sample delivery rather than a directional microfluidic approach for simplicity and reliability—this led to the Chip design already discussed (Figure 1). We thus deliver samples to the dry ChemChips using 14 mm diameter circles of Whatman filters; the filters were clamped onto the center of the ChemChips. 25uL samples were dispensed on the center on the membrane. The membranes hydrate uniformly; less that 1 μL of the 25μL sample is available for each of the 25 wells. Given a 2mm well spacing and a 0.2mm thick membrane, up to 510 nL is delivered to any given well. The sample wicks along the membrane and in to each well whereupon the reagents rehydrate and the bioluminescence reactions begin. An optimum process results in a convex lyophilized reagent surface, which facilitates drawing the samples into each of the wells from the membranes (this process will be more fully optimized in the next several months). The

porous and hydrophilic structure of the lyophilized reagents and filter allowed the sample to uniformly fill the wells without bubble formation.

A commercially available Andor DV-434 CCD was used to take a kinetic series of 30-second exposures of the bioluminescence activity for each assay. Typical CCD Images can be seen in Figure 3.

The analytical results are summarized in Table I and Figures 4-6. The Milestone 2 detection limits were exceeded by at least a factor of 20 for all 4 analytes. Although the average error reported for galactose and lactate exceed 20% in this data, had we not pushed the detection limit (we got carried away), we would have come in well under 20%. A major component of the error is sample volume delivery, which we are now addressing using more controlled means for vertical sample delivery

3) Microdevice Detector Arrays:

This milestone was easily met using the Andor DV-434 CCD (Table 1). We have preliminarily evaluated two much less expensive detector arrays: Hamamatsu S8550 and S7585 photodiode arrays. The assays produced an estimated 10 nanoWatts/steradian/cm^2. Such a signal produces a current signal of about 50 pA (about 50 times greater than the dark current) on the Hamamatsu S8593 and S8550 photodiode arrays (given a collection angle of 1 steradian, an area of 5.3mm^2, and a photosensitivity of 0.3 A/W). Given the results reported above, these new arrays will be adequate and will greatly simplify the detection instrument – they are now under extensive evaluation and are the focus of the work in June and July, 2004).

PHASE II (R33) RESEARCH PLANS

R33 Abstract:

We propose to continue the development of microdevices for the specific chemical analysis of multiple metabolites in small sample volumes of biological fluids. The bioluminescent-based enzyme reactions developed in the R21 Phase will be augmented by bioluminescent immunoassays to greatly extend the clinical applicability of the ChemChip system. The Phase 1 (R21) Milestones were successfully met.

The goal of the second phase (R33) is to evaluate and utilize much less expensive array detectors, implement other bioluminescent assays including immunoassays, develop specific diagnostic panels, utilize practical biofluid samples, enhance analytical accuracy and precision, and develop effective means of presentation of the multi-analyte data to the caregiver and patient.

Specific Aim 1—Analyte/Panel Development—will provide assays for additional important metabolites, nutrients, and therapeutic drugs, including a kidney/hemodialysis panel;

Specific Aim 2 – Blood and Urine Samples – will deal with urine calibration issues, blood separation and calibration, and tear sample analysis and calibration;

Specific Aim 3 – Quality Assurance – will develop means for on board chip calibration, enhance the stability of the chips for long term storage, and deal with range features;

Specific Aim 4 – Data Analysis and Multi-Parameter Visualization – what we commonly call InfoWare –

will develop multi-parameter visualization tools; particularly multi-axes radar plots to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

Figures and Tables

Table 1: Detection Limits and Ranges Achieved for ATP, NADH, Galactose, and Lactate in Lyophilized Bioluminescence-Based Assays

Analyte	Required Detection Range	Detectable Range Tested	Sample Volume (maximum of 1 µL)	Experimental Detection Limit (lyophilized form)	Average Error	Detection Limit required for Milestone 2 (lyophilized assays) (error <± 20%)	Detection Limit required for Milestone 3 (lyophilized assays) (error <± 20%)
ATP	Down to 0.001 mM	Linear from 0.001 to 0.1 mM	0.51 µL	0.51 picomoles	19%	100 picomoles	10 picomoles
NADH	Down to 0.1 mM	Linear from 0.01 to 1.0 mM	0.51 µL	5.1 picomoles	21%	2,000 picomoles	200 picomoles
Galactose	0.020 to 0.090 mM	Linear from 0.1 to 1.0M. Logarithmic from 0.01 to 0.1 mM.	0.51 µL	5.1 picomoles	26%	200 picomoles	20 picomoles
Lactate	0.5 to 2.5 mM	Linear from 0.1 to 10 mM	0.51 µL	51 picomoles	22%	5,000 picomoles	500 picomoles

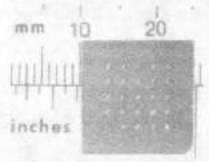


Figure 1: Empty ChemChip

A 5x5 array of 1mm diameter holes were cut from adhesive backed vinyl and sealed to a 15mm square glass cover slip. The notch on the bottom right corner is for indexing the wells.

Figure 2: Reagent Deposition System

A tray holding 25 ChemChips (left) is positioned under a set of miniature solenoid valves with 0.002" nozzles by a computer controlled XYZ stage (right). The tray sits on a copper box filled with dry ice so the assays freeze almost instantly. If the reagents are allowed to thaw or evaporate, the reagent's convex surface collapses resulting less effect sample delivery.

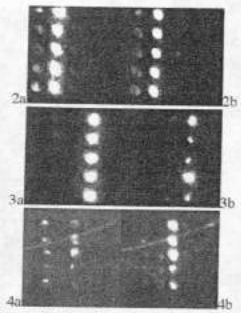
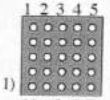


Figure 3: Chemchip Design and CCD Measurements of Bioluminescence Activity



Column	Analyte
1,N	NADH
2,A	ATP
3,L	Lactate
4,G	Galactose
5,B	Blank

1) (right drawing) Bioluminescence assay were dispensed in separate columns in order to get replicate data (5 rows per column).

(left pictures)

2a) NADH and ATP at 1 and 0.1 mM, respectively.

2b) NADH and ATP at 0.01 and 0.001 mM, respectively. This is dimmer than 2a due to lower concentration of analytes.

3a) Galactose assay (1mM sample) at first 30-sec exposure.

3b) Galactose assay (1mM sample) at 6th 30-sec exposure. Being a competition assay, the luminescence decreases with time.

4a) Lactate assay (10mM sample) at first 30-sec exposure. (The streaks of light across the images are due to a cracked cover slip).

4b) Lactate assay (10mM sample) at 6th 30-sec exposure. Being a production assay, the luminescence increases with time.

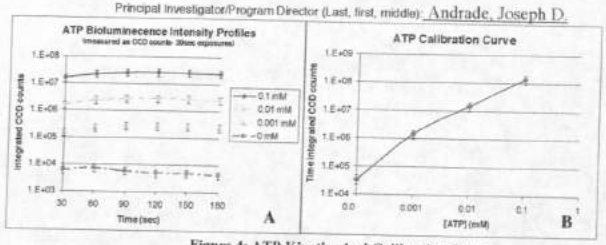


Figure 4: ATP Kinetics And Calibration Curve

25 µL samples containing different concentrations of NADH, ATP, Lactate, and Galactose were delivered to each ChemChip by pipetting the sample onto a 14mm diameter filter membrane clamped onto the ChemChip. Six 30-second exposures were then taken using an Andor DV-434 CCD cooled to -50°C. The CCD counts over the area for each well were then integrated for each time frame. The integrated CCD counts were then averaged across all 5 rows for each column of analyte. An average integration was also taken across multiple chips that were tested at each sample concentration.

A) The intensity time profile for ATP bioluminescence, averaged across multiple chips (n=3) and 5 rows per chip, at 4 concentrations (including a control).

B) This ATP concentration calibration curve calculated by integrating the CCD counts for all six exposures, at each concentration. Given a sample of less than 510 nL, we were able to collect signals that were almost two orders of magnitude greater than the noise level, resulting in a detection limit of 0.51 picomoles. The calibration curve is linear for measurements covering three orders of magnitude.

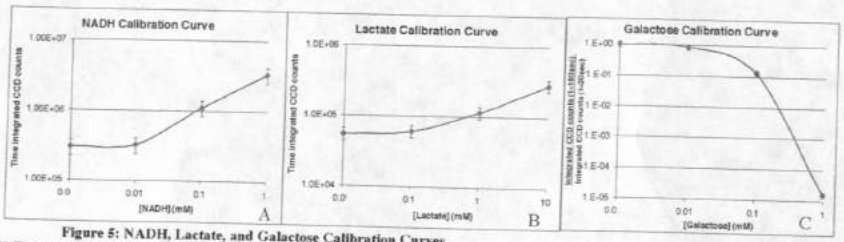


Figure 5: NADH, Lactate, and Galactose Calibration Curves

A) The NADH calibration curve shows a linear response from 0.01 to 1 mM. A 0.01 mM NADH sample (510nL) resulted in a detection limit of 6.4 picomoles. (0.01mMx510nL=5.1picomoles).

B) The Lactate calibration curve shows a linear response from 0.1 to 10nM with a detection limit of 6.4 picomoles

C) Because the Galactose assay was a competition assay, the calibration curve is based on the integrated CCD counts at t=180sec, divided by the integrated CCD counts at t=30sec (the brightest exposure period). This graph shows a linear response between 0.1 and 1 mM. As the Galactose concentration approaches 0.01mM, response is less linear. This non-linear region can be seen in Figure 6, which is the same data in Figure 5C without plotting the Y-axis on a log-scale.

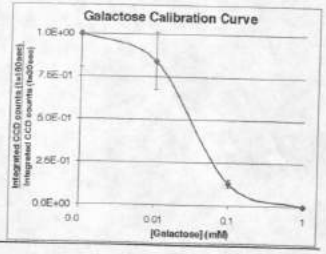


Figure 6 (right): Galactose Calibration Curve (Y-Axis not on Log Scale)
With the Y-axis plotted on a linear scale, we can better see the limits of detection and errors for the Galactose assay as the sample concentration approaches 0.01mM. We estimate a detection limit of 5.1 picomoles (0.01mMx510nL = 5.1 picomoles)

The **Specific Aims** for the R33 Phase II have not been modified from the original application. The details for each of the Specific Aims were presented in the original proposal.

Significance:
The ChemChip system will provide comprehensive diagnostic and monitoring panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs. The development of panels appropriate to specific clinical research areas, such as the kidney/hemodialysis panel, will greatly facilitate such research, due to the ease of use, low cost, and multi-parametric data generation provided by ChemChip devices. When more fully developed and available, ChemChips can be considered a research resource for a wide range of clinical and biochemical studies. A specific example is the thesis now underway by Jensen (see title below), potentially applying ChemChips to endocrine disorders.

Publications (*=copy is attached)
Journal Article:

R. Davies, D. Bartholomeusz, J. Andrade, "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" IEEE Engineering in Medicine and Biology Magazine, Volume 22, Issue 5, Jan/Feb (2003), pg 33-43.(this paper was cited and submitted with last year's Progress Report)

Journal Article (accepted and in press):

*Sang IL Jeon, Xiaoyun Yang and Joseph D. Andrade, "Modeling of Homogeneous Immunoassay (CEDIA)", Analytical *Biochemistry, accepted May, 2004*.Journal Article (submitted):

*Xiaoyun Yang, Jarmila Janatova, and Joseph D Andrade, "Single Step CEDIA using Spectrophotometric, Chemiluminescent, and Bioluminescent Substrates," submitted to J Immunologic Methods, May, 2004.Conference Proceedings:

Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units, D. Bartholomeusz and JD Andrade, in PE Stanley and LJ Kricka, eds., Bioluminescence and Chemiluminescence, World Scientific, (2002), pp. 189-192 (this paper was presented with the last Progress Report)

*Lactate Assay based on Bacterial Bioluminescence: Enhancement, Dry Reagent Development, and Miniaturization, RH Davies, JW Corry, JD Andrade, Ibid., pp. 441-444. (although this paper and the next did not cite support of this grant, they should have, as the work was largely supported by this grant—we apologize for the oversight)

*Enzyme Kinetics Model of the Bacterial Luciferase Reactions for Biosensor Applications, Y Feng, RH Davies, JD Andrade, Ibid., p. 88.

Abstracts:

*Bartholomeusz DA, Davies RH, Al-Sheikh YT, and Andrade JD, "Development of a Multi-Analyte ChemChip for Metabolic Assessment and Monitoring," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 231.

*Abernathy, JB, Davies, R, and Andrade J, ""Bioluminescence-Based Glucose Assay for a Multi-Analyte Biosensor," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 227.

Davies, RH Andrade, JD, "Diabetes-Related Luminescent Assays for Multi-Analyte Measurement" in *Diabetes Technology & Therapeutics*, 2003, **6**(2): p. 237

Abstracts Submitted:

will develop multi-parameter visualization tools; particularly multi-axes radar plots to produce disease and condition specific signatures or patterns, which can be made recognizable by a lay patient population.

The **Specific Aims** for the R33 Phase II have not been modified from the original application. The details for each of the Specific Aims were presented in the original proposal.

Significance:
The ChemChip system will provide comprehensive diagnostic and monitoring panels for basic research, clinical research, and for personal disease and health management. These systems would provide rapid results, facilitate patient empowerment, and reduce health care costs. The development of panels appropriate to specific clinical research areas, such as the kidney/hemodialysis panel, will greatly facilitate such research, due to the ease of use, low cost, and multi-parametric data generation provided by ChemChip devices. When more fully developed and available, ChemChips can be considered a research resource for a wide range of clinical and biochemical studies. A specific example is the thesis now underway by Jensen (see title below), potentially applying ChemChips to endocrine disorders.

Publications
Journal Article:

R. Davies, D. Bartholomeusz, J. Andrade, "Personal Sensors for the Diagnosis and Management of Metabolic Disorders" IEEE Engineering in Medicine and Biology Magazine, Volume 22, Issue 5, Jan/Feb (2003), pg 33-43.(this paper was cited and submitted with last year's Progress Report)

Journal Article (accepted and in press):
*Sang IL Jeon, Xiaoyun Yang and Joseph D. Andrade, "Modeling of Homogeneous Immunoassay (CEDIA)", Analytical *Biochemistry, accepted May, 2004*.Journal Article (submitted):

*Xiaoyun Yang, Jarmila Janatova, and Joseph D Andrade, "Single Step CEDIA using Spectrophotometric, Chemiluminescent, and Bioluminescent Substrates," submitted to J Immunologic Methods, May, 2004.Conference Proceedings:

Photodetector Calibration Method for Reporting Bioluminescence Measurements in Standardized Units, D. Bartholomeusz and JD Andrade, in PE Stanley and LJ Kricka, eds., Bioluminescence and Chemiluminescence, World Scientific, (2002), pp. 189-192 (this paper was presented with the last Progress Report)

*Lactate Assay based on Bacterial Bioluminescence: Enhancement, Dry Reagent Development, and Miniaturization, RH Davies, JW Corry, JD Andrade, Ibid., pp. 441-444. (although this paper and the next did not cite support of this grant, they should have, as the work was largely supported by this grant—we apologize for the oversight)

*Enzyme Kinetics Model of the Bacterial Luciferase Reactions for Biosensor Applications, Y Feng, RH Davies, JD Andrade, Ibid., p. 88.

Abstracts:

*Bartholomeusz DA, Davies RH, Al-Sheikh YT, and Andrade JD, "Development of a Multi-Analyte ChemChip for Metabolic Assessment and Monitoring," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 231.

*Abernathy, JB, Davies, R, and Andrade J, ""Bioluminescence-Based Glucose Assay for a Multi-Analyte Biosensor," *Diabetes Technology & Therapeutics*, 2004, **6**(2): p. 227.

Davies, RH Andrade, JD, "Diabetes-Related Luminescent Assays for Multi-Analyte Measurement" in *Diabetes Technology & Therapeutics*, 2003, **6**(2): p. 237

Abstracts Submitted:

Intern. Conf.on Bio- and Chemi-Luminescence, Yokohama, Sept. 2004:

Bartholomeusz DA, Davis RH, Andrade JD Method for implementing bioluminescence-based analytical assays in nanoliter volumes

Al-Sheikh Y, Abernathy J, Bartholomeusz DA, Davis RH, Andrade JD Multi-parametric experimental design of bioluminescence-based analytical assays

Theses in Preparation:
R Davies, PhD., Dec., 2004: Luminescent Assays for the Assessment of Metabolism and Renal Function: Towards a Multi-Analyte Biosensor.
D. Bartholomeusz, PhD., Spring, 2005: Fabrication of a Bioluminescence-Based Multi-Analyte Biosensor
X.Y. Yang, PhD., June, 2006: Homogeneous Chemiluminescent Immunoassay for Application to ChemChip Devices
Y. Al-Sheikh, PhD., Spring, 2005: Calibration, Data, Visualization, and Interpretation of a Multi-Analyte, Luminescence-Based ChemChip
P. Mohan, MSc., Spring, 2005: Toward a renal chip for assessment of kidney function
J. Jensen, PhD., Dec., 2006: Toward Analysis of Major Steroid Hormones via Personal Multi-analyte Biosensors: Patient Management of Endocrine Disorders.
M. Yang, PhD., June, 2007: Process and Fabrication Issues in the Preparation and Production of ChemChips

Form Approved Through 9/30/2007

OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Progress Report

Review Group	Type	Activity	Grant Number
			5R33RR017329-04

Total Project Period
From: 8/1/04 Through: 7/31/05

Requested Budget Period
From: 8/1/05 Through: 7/31/06

1. TITLE OF PROJECT
Multi-Analyte Micro-Devices for Biomedical Applications

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Name and address, street, city, state, zip code)

Joseph D. Andrade, Ph.D.
50 S. Central Campus Dr. 2480 MEB
Salt Lake City, UT 84112-9202

3. APPLICANT ORGANIZATION (Name and address, street, city, state, zip code)

University of Utah
1471 Federal Way
Salt Lake City, UT 84102

2b. E-MAIL ADDRESS
joeandrade@uofu.net

4. ENTITY IDENTIFICATION NUMBER

2c. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Bioengineering

2d. MAJOR SUBDIVISION
College of Engineering

5. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL

Office of Sponsored Projects: Assistant Director
1471 FEDERAL WAY
SALT LAKE CITY UT 84102

E-MAIL: ospawards@osp.utah.edu

6. HUMAN SUBJECTS
☒ No ☐ Yes

6a. Research Exempt ☐ No ☐ Yes

6b. Human Subjects Assurance No.
FWA00003745

If Exempt ("Yes" in 6a):
Exemption No.

6c. NIH-Defined Phase III Clinical Trial ☒ No ☐ Yes

If Not Exempt ("No" in 6a):
IRB approval date
☐ Full IRB or ☐ Expedited Review

7. VERTEBRATE ANIMALS
☒ No ☐ Yes

7a. If "Yes," IACUC approval Date

7b. Animal Welfare Assurance No.
A3031-01

8. COSTS REQUESTED FOR NEXT BUDGET PERIOD

8a. DIRECT $

8b. TOTAL $

9. INVENTIONS AND PATENTS
☐ No ☒ Yes If "Yes," ☐ Previously Reported ☒ Not Previously Reported

10. PERFORMANCE SITE(S) *(Organizations and addresses)*

University of Utah
50 S. Central Campus Dr.2480 MEB
Salt Lake City, UT 84112-9202

11a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR *(Item 2a)*
Andrade, Joseph D.
TEL 801 581-4379
FAX 801 585-5361

11b. ADMINISTRATIVE OFFICIAL NAME *(Item 5)*
Vincent A. "Bo" Bogdanski
TEL 801 581-3008
FAX 801 581-3007

11c. NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION *(Item 14)*
NAME Elliott C. Kulakowski, PhD
TITLE Director, OSP
TEL 801-581-6903 FAX 801-581-3007
E-MAIL ospawards@osp.utah.edu

12. Corrections to Page 1 Face Page

There are no changes to the direct and total costs requested for the next budget period.

13. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.	SIGNATURE OF PI/PD NAMED IN 2a. *(In ink. "Per" signature not acceptable.)*	DATE
14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 11c. *(In ink. "Per" signature not acceptable.)*	DATE