AA

Form Approved Through 2/28/01
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK--FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT
Biosensors for Chronic Biochemical Diseases

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES *(If "Yes," state number and title)*
Number: PAS-00-006 Title: Bioengineering Research Partnerships

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR
New Investigator ☐ YES

3a. NAME *(last, first, middle)*
Andrade, Joseph D

3b. DEGREE(S)
Ph.D.

3c. SOCIAL SECURITY NO.
Provide on Form Page KK.

3d. POSITION TITLE
Professor

3e. MAILING ADDRESS *(Street, city, state, zip code)*
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3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Bioengineering

3g. MAJOR SUBDIVISION
College of Engineering

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (801)581-4379
FAX: (801)585-5361

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4. HUMAN SUBJECTS ☐ No ☒ Yes
4A. If "Yes," Exemption no. or IRB approval date 12/21/99 { ☒ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ☒ No ☐ Yes
5a. If "Yes," IACUC approval date
5b. Animal welfare assurance no.

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	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
5/1/01	4/30/06	488,957	708,436	2,342,075	3,488,114

9. APPLICANT ORGANIZATION
Name University of Utah
Address 1471 Federal Way
Salt Lake City, Utah 84112

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE 20

12. ENTITY IDENTIFICATION NUMBER
876000525
DUNS NO. (If available)
00909-5365

Congressional District
02

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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15. 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 the 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 IN 3A. *(In ink. "Per" signature not acceptable.)*
DATE 8/8/00

16. 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 Service 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 14. *(In ink. "Per" signature not acceptable.)*
DATE 8/8/00

CERTIFICATION PAGE

Certification for Principal Investigators and Co-Principal Investigators:

I certify to the best of my knowledge that:

(1) the statements herein (excluding scientific hypotheses and scientific opinions) are true and complete, and
(2) the text and graphics herein as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or individuals working under their supervision. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if an award is made as a result of this application.

I understand that the willful provision of false information or concealing a material fact in this proposal or any other communication submitted to NSF is a criminal offense (U.S.Code, Title 18, Section 1001).

Name (Typed)	Signature	Date
PI/PD J.D. Andrade	[signature]	5/13/93.
Co-PI/PD		
Co-PI/PD		
Co-PI/PD		
Co-PI/PD		

Certification for Authorized Institutional Representative or Individual Applicant:

By signing and submitting this proposal, the individual applicant or the authorized official of the applicant institution is: (1) certifying that statements made herein are true and complete to the best of his/her knowledge; and (2) agreeing to accept the obligation to comply with NSF award terms and conditions if an award is made as a result of this application. Further, the applicant is hereby providing certifications regarding Federal debt status, debarment and suspension, drug-free workplace, and lobbying activities (see below), as set forth in Grants for Research and Education in Science and Engineering (GRESE), NSF 92-89. Willful provision of false information in this application and its supporting documents or in reports required under an ensuing award is a criminal offense (U. S. Code, Title 18, Section 1001).

Debt and Debarment Certifications

(If answer "yes" to either, please provide explanation.)

Is the organization delinquent on any Federal debt? Yes ___ No ___

Is the organization or its principals presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency? Yes ___ No ___

Certification Regarding Lobbying

This certification is required for an award of a Federal contract, grant, or cooperative agreement exceeding $100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding $150,000.

Certification for Contracts, Grants, Loans and Cooperative Agreements

The undersigned certifies, to the best of his or her knowledge and belief, that:

(1) No federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned, to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.

(2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned shall complete and submit Standard Form-LLL, "Disclosure Form to Report Lobbying," in accordance with its instructions.

(3) The undersigned shall require that the language of this certification be included in the award documents for all subawards at all tiers including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by section 1352, title 31, U.S. Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

AUTHORIZED INSTITUTIONAL REPRESENTATIVE		SIGNATURE	DATE
NAME/TITLE (TYPED) R. Koehn, V.P. for Research			
TELEPHONE NUMBER 801-581-7236	ELECTRONIC MAIL ADDRESS	FAX NUMBER 801-585-6212	

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 first person. This description 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.

Chronic illness is a major and growing contributor to health care expenses in the USA. Biochemical testing is central to the management of many chronic diseases and conditions. Today such testing generally requires relatively large fluid volumes and is done in hospital or clinical chemistry laboratories. The cost of testing and the delay in receiving the needed results often constrains timely health care decisions. There is also a need to measure a range of analytes in order to more fully study, understand, characterize and manage the complex biochemistry involved in major chronic diseases. We propose to develop home and point of care biochemical analysis technologies for the monitoring and study of patients with chronic conditions. The sensors, called ChemChips, will have multiple analytical channels for metabolites.

Metabolic/biochemical modeling and simulation, and extensive clinical input, will insure that appropriate and relevant metabolites are measured, both for clinical disease management and for clinical research. Multivariate analyses and multi-dimensional visualization will be used to present the information in an efficient and effective manner, intelligible to physicians and nurses as well as to untrained but educated and empowered patients and family members.

Years 1-2 build on existing activities by focusing on the chronic metabolic diseases phenylketonuria (PKU) and galactosemia; years 2-3 focus on epilepsy, particularly the ketogenic diet; years 4-5 address depression and related mental states. The ChemChip will begin as a 10 channel device, developing into a 50 channel (30-40 analyte) ChemChip by Year 5. It will also be applicable to other chronic biochemical diseases and conditions. This BRP project includes a sensor/analytical chemistry group; a simulation and visualization group; a clinical group; a National Advisory Board; consultants in n-dimensional visualization, luminometer instrumentation, and micromachining; and a corporate collaborator.
This is a revised application (R01-DK-58507-01).

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

University of Utah — Salt Lake City, Utah

KEY PERSONEL. See instructions on Page 11. Use continuation pages as needed to provide the required information in the format shown below.

Name	Organization	Role on Project
Joseph D. Andrade, Ph.D.	University of Utah	PI
Steven E. Kern, Ph.D.	University of Utah	Task Leader
Edward B. Clark, M.D.	University of Utah	Task Leader
Dana Hardin, M.D.	University of Utah	Investigator
John Holman, M.D.	University of Utah	Investigator
James Wiskin, Ph.D.	University of Utah	Investigator
Jarmilla Janatova, Ph.D.	University of Utah	Investigator
Russell Stewart, Ph.D.	University of Utah	Investigator

Partnership Organization:

The Univ. of Utah is the lead institution, with consultant participation from Georgia Tech (B. Frazier) and n-DV, LLC (R. Johnson). Univ. of Utah participating units include the Dept of Bioengineering (J. Andrade, R. Stewart, J. Janatova), Pharmaceutics (S. Kern), Surgery (J. Holman), Pediatrics (E. Clark and D. Hardin), Biomathematics (J. Wiskin).

Joe Andrade, Professor and co-chair of the Dept. of Bioengineering is the PI and leader of Tasks 3 and 4. Steve Kern, Asst. Professor of Pharmaceutics, leads Tasks 2 and 5. Ed Clark, Professor and Chair of the Dept. of Pediatrics and Medical Director of the Primary Children's Medical Center, leads Task 1. These three serve as an Executive Committee which meets weekly to plan and review the overall project. The individual task groups meet regularly, and the entire BRP project team has a monthly technical review meeting. Fig. 1 presents the overall organization. The main BRP office will be in the Dept. of Bioengineering.

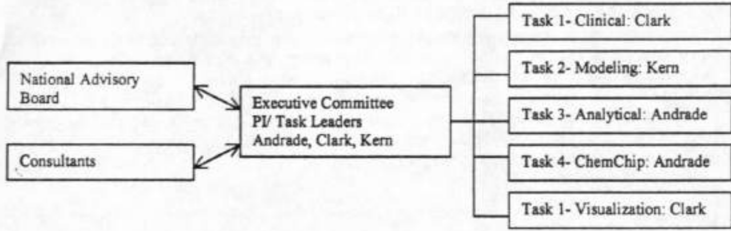


Figure 1 Project Organization

An Advisory Board for the project will likely include B. James, P. Lee, J. Winters, C. Jaffe, O. Ash, N. Waitzman, R. Huefner, P. Mendes, F. Cerra, E. McCabe, G. Stephanapoulos. The Board will meet annually in Salt Lake City to review plans and progress.

Dr. R. Johnson, emeritus chair of the Dept. of Computer Science, University of Utah, and founder and manager of nDV, LLC, will consult in the area of n-dimensional visualization, presentation, and data analyses. Dr. Bruno Frazier, Asst. Prof. Electrical Engineering at Georgia Tech, will consult in the area of micromachining. Their backgrounds, activities, biosketches, and letters are described in Section I: Advisors and Consultants.

Fig. 2 presents the five key tasks and their inter-relations; Fig. 3 is the time plan from several different perspectives.

Budget Justification (Cont.)

Dr. Bruno Frazier, Assistant Professor of Electrical Engineering at Georgia Tech and previously Assistant Professor of Bioengineering at the University of Utah, will participate as a consultant in the micro-machining area (task 4); he is budgeted for five days at $1000 per day, and this includes his travel and local expenses. His letter of agreement and biosketch are also in Section I.

Dr. Richard Van Wagenen, previously VP of R and D for Protein Solutions, Inc. and now an independent consultant, will consult on luminescence detection and instrumentation for 6 days at $1,000/day. His letter of agreement and biosketch are also in Section I.

We had considerable success with a national advisory board for our National Science Foundation/Whitaker Foundation project on Cost Reducing Health Care Technologies (CRHCT), which provided the resources for the preliminary work which led to this application. We propose to establish a National Advisory Board, consisting in part of the members of the previous board, and including other distinguished individuals with particular expertise and skills in the various project areas. This group will provide objective perspective, analysis, and critique of our plans and our activities. The Board will meet annually in Salt Lake City; each board member is budgeted for one day at $1000, of which $500 is a consulting fee and $500 is for expenses.

Major Equipment (defined as greater than $5,000 cost) is budgeted at $50,000, primarily to help establish the recombinant enzyme facility. The equipment items include a refrigerated ultracentrifuge, a UV-visible spectrometer, and a high-pressure liquid chromatograph.

Supplies costs are $57,000 in the first year, decreasing to $37,000 in Year 5. Supplies also include small equipment items (< $5000) as well as needed consumables:

molecular biology supplies	15,000
commercial enzymes andother proteins	9,000
glassware and plasticware	7,000
chromatography and electrophoresis supplies	12,000
solvents and biochemicals	5,000
computer software and licenses	4,000
other materials and electronic components	5,000

Travel costs are for the annual BRP grantees' meeting and for participation in technical conferences and workshops; we have budgeted 5 such trips, including registration fees, travel expenses, and lodging.

Other expenses are for analytical services, primarily for our local regional clinical chemistry laboratory, ARUP (www.arup-lab.com); this is to provide clinical chemistry testing and evaluation services with which to assess, evaluate, and calibrate our analytical devices. The other entry is for micro-machining services at the University of Utah and Georgia Tech.

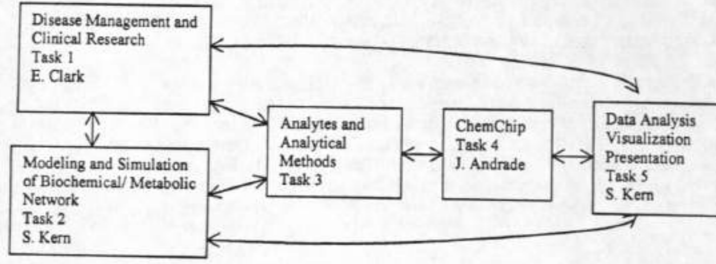


Figure 2 Task Connections and Relationships. Tasks 1 and 2 identify the most useful and interesting analytes (about 25 are already well defined). Task 3, with the help of Task 2, identifies appropriate enzyme analytical methods for the analytes, develops and tests the assay, and transfers it to Task 4. Tasks 4 implements the assay as one or more channels in the ChemChip (one analyte may require more than one channel due to standardization and/or calibration needs). Task 5 uses the recommendations and input from Tasks 1, 2, and 4 to optimally analyze, visualize, and present the information. Tasks 3 or 4 may conclude that the measurement of a specific analyte may be impractical; we will then look to Task 2 (and 3) to identify a different, more readily measured, analyte which could provide comparable information.

Time/ Year		1	2	3	4	5	6-10
Disease or Condition	PKU/ Gal						
	Epilepsy						
	Depression						
	other						
Tasks	1. Clinical						
	2. Modeling						
	3. Analytical						
	4. ChemChip						
	5. Visualization						
Analytes/ Channels	10						
	20						
	30						
	40						
	50						

Figure 3 Time Plan and emphasis from several key perspectives Dotted lines indicate reduced emphasis (allocation of post doc, technician, and graduate student resources). We plan to develop about 10 channels per year, achieving a total of 30 – 40 analytes by Year 5 (control and calibration channels are also needed).

RESEARCH PLAN (Abbreviations, Web Site addresses, and some definitions are listed for convenience at the end of Section I: Literature Cited)

Specific Aims and Tasks

The overall aim is to develop and test biosensors which permit multi-analyte, multi-channel biochemical measurements relevant to chronic biochemical diseases in the point of care or home environment. Emphasis is on very low volume blood and urine sample sources which minimize patient discomfort and inconvenience (thus maximizing compliance). A further goal is to deliver and effectively present multi-analyte data to the patient, his/her family, and the health care provider using means and methods which enhance the multi-variate relationships and understanding of the condition, thereby enhancing patient/provider empowerment and disease management.

The project is based on three fundamental **assumptions:**

1) relevant information, appropriately and effectively presented, empowers the patient, the family, and the physician (Task 1 and the output of Task 5);
2) nearly all pathologies have biochemical origins and consequences (Tasks 1-4);(1, 2)
3) nearly all biochemicals could be quantitatively and specifically measured in small volume samples via relatively inexpensive, reliable instruments in the point of care or home environment (Tasks 3,4).

There are a number of **dimensions and perspectives** to the project (please refer back to Figures 2 and 3):

1) the disease, pathology, clinical need and clinical research perspective (Tasks 1,2) — PKU/galactosemia, epilepsy, depression;
2) the analyte perspective (Tasks 1-5) – amino acids, vitamins, secondary metabolites, etc.;
3) the biochemical modeling and simulation perspective (Task 2) – to enhance the appreciation and understanding of the multi-variate relations and for hypothesis development;
4) the presentation/visualization perspective (Task 5) – to effectively and efficiently present important information in an easily comprehended form at several educational and "need" levels: patient, family or immediate caregiver, nurse, physician.

Task 1 — Disease Management and Clinical Research Needs -- Task Leader: E. B. Clark

There are many diseases and clinical conditions for which the need for regular, routine clinical chemistry measurements are well recognized. Glucose measurement for the management of diabetes is well developed. There is general acceptance of the need for monitoring phenylalanine and tyrosine for the management of phenylketonuria (PKU) and galactose and galactose-1-phosphate for the management of galactosemia, but home assays are not available. The monitoring of creatinine as a measure of kidney function in transplant patients is well known, but a home assay is not available. The management of the ketogenic diet for certain types of epilepsies is also recognized, but means to regularly and inexpensively monitor the metabolic consequences of the diet are not available. There is considerable interest in the role of diet and individual biochemical differences in brain metabolism and mental states (Siegal), but little data, nor inexpensive means to obtain such data, are available.

In this task we continue to identify the analytes needed for the optimum management of the disease or condition. Another major part of this task is to facilitate clinical research by working closely with Task 2, considering the overall biochemistry of the disease or condition, hopefully leading to novel and specific new hypotheses, a more complete understanding, and to better treatment modalities.

Task 2 – Modeling and Simulation of Biochemical Networks -- Task Leader, S. Kern
Metabolism involves literally thousands of substrates, enzymes, and products – mutually interacting in a complex set of biochemical networks. For example, the enzyme defect in PKU leads to far more than the elevation of Phe – it leads to decreases in Tyr and to changes in the concentrations of Val, Leu, iLeu, and Met. Phe and Tyr biochemistry couples to the production of DOPA, dopamine and epinephrine, likely related to some of the mood and emotional conditions often associated with PKU. In galactosemia the elevated galactose levels drive several other biochemical pathways which produce galactitol and galactolactone, which may be the primary "toxins" in galactosemia.

It is now practical to model and simulate complex biochemical networks and to assess the effect of a change in one metabolite on the concentration of other coupled metabolites. Such computer experiments allow us to predict the key metabolites for which assays should be developed and studied. It is important to point out that initially we will use "soft", qualitative modeling (3);as more is learned about the pathways, particularly key concentrations and enzyme reaction parameters, the models will become progressively more quantitative and useful in clinical research and in the management of disease conditions.

Modeling and simulation also allow us to assess the practicality of proposed new assays, permitting the most practical enzyme assay to be selected for initial study and development (Task 3).

Task 3 – Analytes and Analytical Methods --Task Leader, J.D. Andrade

We will utilize bioluminescence-based specific enzyme assays (4) to provide the sensitivity, specificity, and simplicity needed for a multi-analyte biosensor for eventual home use:

firefly luciferase/ATP based enzyme assays for one set of metabolites and
bacterial luciferase/NADH based assays for another set.

We will develop assays for the metabolites identified in Tasks 1 and 2. The goal is to develop individual analyte channels, utilizing initially only 1 to 2 microliters of blood for each channel, eventually allowing up to 50 different measurements providing data for 30 to 40 analytes from a single 50 microliter sample.

This project does not address the measurement of analytes which require immunoassay, HPLC, electrochemical, or other methods for optimum analyses. Clearly, therapeutic drug monitoring (normally by immunoassay or HPLC), ions, and other analyses could and should be included in future projects so that a futuristic ideal ChemChip would include all analytes and ions of importance to the clinician and patient.

Task 4 – The General ChemChip -- Task Leader, J.D. Andrade

The analyte channels now available to us, plus the additional ones we will develop, can all be incorporated into a general analytical device we tentatively call the ChemChip. It will eventually measure some 30 to 40 different analytes using a small luminometer. The luminometer will perform on board data analysis and use novel means (Task 5) to present the information in a

Response to Summary Statement and Critique of RO1 DK-58507-01 (Dr. C. McKeon , 301-594-8810)

The review was generally positive with respect to vision, significance, innovation, and need but noted weaknesses in "...this ambitious project". During the preparation of this revision the comments, critiques, and suggestions of the reviewers have been addressed in the appropriate sections.

We have eliminated the previous task on alternate sample sources (tears, etc.), the former Task 4. We have eliminated the immunoassay and therapeutic drug modeling work (in Task 3). We have removed the work on structured, organized multi-enzyme complexes (also in Task 3), as we have learned that the increased efficiency and sensitivity they might provide are not really needed for most of the analytes we propose to study. We have eliminated the work on a conventional paper-based dipstick sensor, focusing the activity on the ChemChip micro-fabricated sensor (now Task 4); this work now begins in Year 1 and is discussed in more detail in this revised application. We have made significant progress in the ChemChip area since the original submission some 8 months ago.

The role of, and need for, the modeling and simulation work has been now addressed and discussed in more detail.

We have addressed the concern that (to paraphrase the reviewer): "...the clinical research should be done first, the needed analytes identified, and **then** the sensors developed." We agree only in part; we have now explained in the proposal that inexpensive multi-analyte sensors are required to enable and empower the biochemical clinical research that is needed for better understanding and treatment of biochemical diseases..

Background and Significance

Although clinical chemistry and analytical laboratories can routinely assay carbohydrates, amino acids, vitamins, and other low molecular weight biochemicals important to metabolism, in practice most of the major metabolites are rarely determined. One would think that the circulating plasma levels of the 20 major amino acids, for example, would be easily and readily measured, given today's technologies and analytical capabilities. This is not the case. It is rare for a physician or a nutritionist to order a total amino acid analysis, because it is expensive and difficult to interpret. Indeed the direction in clinical medicine has been to order less chemical tests rather than more. This of course has to do with the interest in minimizing health care costs and thus in minimizing "unnecessary tests". The expense of most tests, given typical cost-benefit analyses, argues against more comprehensive biochemical analyses. Such conclusions are based on the assumption that the costs of such tests will not decrease. If the costs for biochemical testing were significantly lower, the cost-benefit analyses would be different, likely leading to recommendations for more rather than fewer tests.

We propose to develop means to provide multi-analyte biochemical testing at costs far less than the cost of a single test today.

We will soon have the means with which to assess our genetic makeup and therefore our susceptibility to genetic diseases (Nature special issue June 15, 2000 and Scientific American July, 2000 special issue). In the next decade or two we will also know the result of that genetic coding, our specific individual proteome. But the output of much of the proteome -- our specific

useful, interpretable format . Eventually a hand-held (for example, PalmPilot or Handspring Visor) unit can be used with a hand-held luminometer to transfer the information to the health care provider via E mail or the Internet. Such instrument development is not a part of this project in Years 1-5. We will, however, further develop our existing portable CCD camera/laptop computer based luminometer in this project.

The initial development of the Micro-Electromechanical System (MEMS) multi-analyte ChemChip will focus on the miniaturization and stabilization of the bioluminescent reactions within microfabricated reaction channels. Micromachining techniques will be applied to create:

- light enhancing features and devices within bioluminescent reaction channels in the ChemChip in order to increase the signal per sample volume efficiency ratio,
- microfluidic channels that allow for efficient and accurate sample filling by capillary action without bubbles or clogging,
- smooth integration of the ChemChip with linear and two dimensional CCD for simple instrumentation and low-cost detection,
- placement preparation and long-term stabilization of multiple enzymes in analyte specific chambers for repeatable measurements,
- tests with blood (filtering and accounting for protein adsorption and clogging) and
- quality assurance features to test accuracy and stability of enzymes. Silicon wafer etching and poly(dimethylsiloxane) (PDMS) molding will be used and compared as means for rapidly creating prototype ChemChips with channels and branches of varying shapes and sizes (each channel holding about 10 to 100-nL

In Year 1 a 10 channel ChemChip will be developed and tested; in Years 2-3 on board blood cell filtering and Hematocrit determination will be included; by Year 3 the Chip will contain 20 analytical channels. By Year 5 the final ChemChip will include 50 channels. A 50 microliter blood sample is assumed for Years 1 through 5.

Task 5 -- Data Analysis, Visualization, and Presentation -- Task Leader, S. Kern

It is generally difficult to deal with many different channels of information. Therefore, we address what is sometimes called the "cockpit problem" – how does a pilot, for example, deal with the tens and even hundreds of 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 up to 40 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.

We will experiment with the novel approaches of nDV, LLC, a local company whose focus is n-dimensional visualization (go to www.globalsvcs.com/hdv^ -- please treat this as proprietary information). We will also experiment with a range of other multi-parameter visualization tools, particularly multi-axes radar plots (also called spider, radial, or Tatra plots) (5, 6) (7) to produce disease and condition specific icon-like patterns which are easily recognizable.

The relations between the five tasks and Time Line are in Figs. 2 and 3.

individual metabolism -- is virtually inaccessible today. Our specific, individual metabolism -- our biochemical individuality -- has been called the metabolome.

The measurement of low molecular weight metabolites relies almost exclusively on the separation methods high-pressure liquid chromatography (HPLC), mass spectrometry (MS), and gas chromatography (GC), generally requiring relatively expensive equipment and trained operators. With the practical exception of glucose, inexpensive means for the specific, quantitative, and highly reliable analysis of key low molecular weight metabolites are not readily available. Because there are literally millions of diabetics who must monitor glucose at least several times per day, there has been an economic incentive for the development of reliable, quantitative sensors and simple, inexpensive glucometers for the measurement of glucose. This development has been made possible by the strong commercial incentive, but also because glucose is easy to measure, as it is present in high concentration (millimolar). The measurement of other key metabolites, including nearly all of the amino acids, generally requires more sensitive means of analysis. The relatively simple colorimetric and electrochemical methods used for the analysis of glucose and several other analytes of wide interest (cholesterol, lactate) are generally not sensitive enough for the analysis of most other metabolites, many of which are in the micromolar range.

The diabetes community is leading and driving major research and development activities to further improve the measurement and monitoring of glucose and of other metabolites important to diabetes, with an emphasis on sampling methods which minimize discomfort. Considerable research and development is now being focused upon minimally invasive and small volume methods (www.jdfcure.com and www.diabetes.org). It is likely that these efforts will be successful and that truly, minimally invasive, relatively painless means for acquiring samples for biochemical analysis will become available in the very near future.

But a serious problem with minimally invasive approaches to sampling is that the volume collected is often one microliter or less, presenting a considerable challenge for current methods of measurement. Metabolites whose circulating blood level or urine level is in the micromolar range or higher can be readily measured by bioluminescence assays, using less than 50 microliters of sample.

The PKU Example:

There has been growing awareness that PKU requires regular self-monitoring to ensure dietary compliance and facilitate diet adjustments (8). The management and treatment of PKU has many similarities to diabetes. The problem is that there are no simple, inexpensive means to measure Phe in the home environment. The availability of a home test device would greatly improve the monitoring and management of PKU:

"...A satisfactory diet compliance with ideally low blood Phe concentrations can only be obtained if the principle of frequent monitoring...of blood Phe by the patient himself (self-monitoring) is realized." (8) (9)

Although McCabe and co-workers (10) proposed a home device for Phe "estimation" some ten years ago, they were disappointed that there was no commercial/industrial interest in manufacturing and selling such a device. They noted that:
"A simple, portable monitoring system would provide families and their local physicians with an estimate of the blood Phe concentration within an hour of obtaining the specimen. This might be especially useful in attempting to moderate rising Phe concentrations during intercurrent

illnesses. Home or office monitoring should not replace the clinical laboratory, but would supplement the traditional process by providing more rapid and frequent Phe estimates. Another use...would be to provide information for the maintenance of metabolic control in pregnant women with PKU... maintain strict metabolic control throughout the pregnancy."

The major reason for the lack of commercial interest is the relatively small market. PKU is a low incidence (~1/10,000) "orphan" disease. The costs of developing a home sensor for PKU alone are simply too high to merit commercial interest. This statement should not be confused with the situation and market for newborn screening for PKU and galactosemia. Here the number of births are sufficient and several point of care analyzers exist and are widely used, generally in state and regional screening laboratories.

PKU is difficult to manage since phenylalanine is an essential amino acid. PKU patients should be tested at least weekly for phenylalanine to empower them to maintain tight dietary control. There is considerable clinical concern with dietary compliance and management. This has been most recently recognized in a series of recent studies (9) which demonstrated that if patients "see" their phenylalanine levels regularly, they voluntarily maintain stricter, more effectve dietary management. PKU should be managed similarly to the management of chronic diabetes. (8)

It is difficult today for the biochemical and clinical research communities dealing with relatively rare diseases to make rapid progress due to the difficulty in accessing suitable numbers of patients. The patient issue could be partially alleviated by the availability of simple, minimally invasive, inexpensive sensors which patients and their care-givers could routinely use to monitor and manage their disease and thereby provide a substantive increase in the amount of biochemical data for the research community. The availability of the internet has allowed much of the PKU community (www.pkunews.org) and the galactosemia community (www.galactosemia.org) to organize and interact. This will enhance the involvement and empowerment of these communities in participation in research that will hopefully lead to better understanding, treatment and outcome for these orphan diseases.

Another Example -- Kidney Transplants:

The most useful index of overall renal function is the glomerular filtration rate (GFR), normally determined by creatinine clearance measurements. Significant decreases in urinary creatinine excretion result in an increase in the plasma creatinine level and is indicative of serious renal dysfunction. Significant reduction in renal function over a several week period is defined as acute renal failure. Chronic renal failure is defined as a permanent and significant reduction in GFR and a consequent significant increase in serum or plasma creatinine eventually leading to end stage renal disease (ESRD). Patients suffering from ESRD are generally treated by hemodialysis, peritoneal dialysis, or by renal transplantation -- the most effective therapy. A functioning renal transplant removes endogenous waste products more effectively than dialysis and provides a physiologic source of endocrine products produced by the kidney. Currently 85-90% of renal transplants survive at least one year with a survival half time of 8-15 years after that, depending on the kidney source.

Long term survival of renal transplants is however limited by the host immune response to the donor organ. Patients must take medications to suppress the host immune response. Because of the multiple pathways that the immune system can take in responding to the allograft, the patient usually takes a "cocktail" of medications each of which works to inhibit a different immune pathway. There are a variety of immunosuppression drug protocols; most protocols

substantiated early reports, the persistent questions of how and why the diet works remain just as puzzling now as earlier in the century...there is still no consensus as to the mechanism of the ketogenic diet's antiepileptic effect." (22) (23) (24)

Anticonvulsant drugs are widely used (20) but have a variety of side effects and must be carefully monitored and managed. Epileptic children treated with valproic acid, for example, generate carnitine deficiencies and high ammonia levels. Their serum amino acid levels are also significantly altered. There is some speculation that the actions of valproic acid may actually have some similarity to those of the ketogenic diet. There is a firm link between activation of the excitatory amino acid transmitter system and the generation and propagation of seizure activity.(25)

Key biochemicals relevant to epilepsy and the ketogenic diet have been identified. The next steps are to decide which should be measured (Tasks 1 and 2), to develop the reactions and methods needed for sensing (Tasks 2 and 3), and to present the output of the sensor in a useful manner (Task 5).

A Longer Range Example – Depression, Mental Health, and Amino Acids

There is considerable interest in modern scientific and medical approaches to mental health problems, particularly depression and related states. There is a long history that amino acids, other nutrients, and other biochemical indicators and measures of mental state may indeed have potential for diagnostic and therapeutic purposes. There is a rich literature on the control of brain neurotransmitter synthesis by the availability of biochemical precursors (primarily amino acids) via the patient's nutritional state.(26) There is very recent work showing bimodal seasonal patterns in the availability of plasma tryptophan, for example, that matches seasonal patterns in depression and the prevalence of suicide in the local population.(27) The same group noted disorders in the metabolism of serotonin and noradrenaline with regard to post traumatic stress disorder and depression and the availability of tryptophan, the precursor of serotonin, and tyrosine, the precursor of noradrenaline.(28) There is a long history on the correlation of brain serotonin levels and the neurobiology of depression and there are also strong indications that plasma tryptophan levels do indeed relate, although clearly not in a direct or simple manner, with serotonin biochemistry.(29)

In addition to the more traditional essential amino acids, the historically non-essential amino acids may be conditionally essential under certain conditions, such as in trauma and major surgery.(30) (31) (32) (6) (33) Once obscure or ignored amino acids are now being rediscovered. A good example is taurine.(34) It is quite clear that amino acid biochemistry can be regulated by nutrition and diet in many circumstances. PKU is perhaps the clearest example, but, as we noted earlier, there is far more to PKU than simply phenylalanine and tyrosine. PKU impacts all of the other amino acids.(35, 36) (37) The composition of amino acids in parenteral solutions is also undergoing significant reevaluation. Glutamine, for example, is now being considered a conditionally essential amino acid, particularly in trauma, major surgery, sepsis, and related clinical situations which put a major stress on the patient.(32, 38)

The work of Cerra and coworkers has demonstrated the importance of amino acid levels in severe trauma and related conditions.(32) Cerra, et al. have also pioneered the use of radial multi-analyte concentration plots for the presentation of important metabolite/nutrient information.(30)

include cyclosporine A (CsA) or tacrolimus (FK 506). Therapeutic blood monitoring (TDM) is used to individually tailor the patient's medication.

A rise in serum creatinine of 30 microM from baseline is generally significant and prompts more detailed investigation into the possibility of rejection. Similarly blood samples are taken for determination of immunosuppressive drug level. The monitoring frequency is decreased after the acute rejection period (one year) is past. Compliance with monitoring limits to some degree its effectiveness at detection of rejection episodes. The reduced frequency of creatinine and drug monitoring is a compromise between the need for monitoring to detect possible rejection, costs, and the inconvenience of going to the lab for blood draws. As regular renal function monitoring can help avoid irreversible transplant damage, there is need for a simple, convenient, accurate method of determining renal function that can be performed by patients at home.

Epilepsy:

Epilepsy afflicts 2 to 3 million people in the United States and approximately 40 to 100 million people worldwide. The annual incidence is about 50/100,000with the highest incidence among persons younger than 5 years or older than 65 years; approximately 1 in 1000 individuals in these age groups are afflicted. The total cost in the United States is estimated at 15 to $20 billion.

Abnormalities in plasma amino acid levels occur in patients with various epilepsies.(11) (12) (13) (14) There has been considerable interest in the research literature in the measurement of the plasma levels of several major amino acids, as well as taurine. (15) There is evidence that seizure patients have increased glutamine, decreased lysine and phenylalanine, and other amino acid changes.(16) Whether these chemical effects are the cause of or the result of seizures remains to be determined.

Ketogenic diets are effective alternative therapies for intractable epilepsy.(17) They are high-fat, low protein, low carbohydrate diets used for the treatment of difficult to control seizures. The diet is carefully and individually calculated and rigidly controlled. It was originally developed in the 1920s and designed to mimic the biochemical changes associated with starvation. (18) The diet produces a state of chronic ketosis, resulting in a change in brain energy metabolism and significant changes in plasma and brain concentrations of ATP, pyruvate, lactate, hydroxybutyrate, and other analytes.(19) Although the diet results in major changes in bioenergetics and in brain chemistry, the actual mechanism related to seizure abatement is unknown. Generally about half of the patients put on the diet substantially benefit from the diet. In some cases the benefits are dramatic, as documented in the recent Meryl Streep film, First Do No Harm. Although providing considerable benefit in significant populations, the diet can also have significant side effects. Treated patients may experience reduction in bone mass. Carnitine is apparently depleted, and carnitine supplementation has been recommended.(20) (21)

Growing numbers of patients are being placed on the diet;(22) clinicians remain unsure about the best way to screen patients for placement and are unsure as to how best to monitor them from a metabolic and clinical chemistry perspective. There are inadequate data with which to formulate guidelines:
"these are exciting and challenging times for those interested in the ketogenic diet. From the back burner of anticonvulsant therapy to a well accepted alternative, interest in the ketogenic diet has blossomed in the past several years. While recent studies of clinical efficacy have

There is growing realization that there are considerable interindividual differences in the circulating level of many biochemicals, metabolites, and nutrients. We each have a biochemical individuality.(39) (40) The normal concentration distribution for individual amino acids can be very broad; a normal physiological variation in adults is generally at least +/- 25 % of the mean value and can be as high as +/- 50%, depending on the amino acid measured [Vogt, 1987 #303]. About 15 % of a normal population have circulating tyrosine values nearly two times that of the mean. The situation is comparable in the case of Phe, with a significant hyper Phe population. We really do not know what is the medical and health significance of significantly low or significantly elevated levels of essential or near essential amino acids. The experience with extensive monitoring of PKU populations indicates that average values three times normal can result in measurable changes in performance on neuropsychological tests.(41) What this means for our purposes is that the monitoring of hyper Phe levels, even those below the normal PKU diagnosis threshold, may be of interest and importance to larger populations than only those afflicted with PKU.

It is now generally accepted that brain chemistry is indeed more sensitive to plasma nutrient and neurochemical precursor levels than other organs and tissues. This means that hypo- or hyper-analyte conditions are likely to have some effect on mental function even if there are no obvious clinical effects on other organs or tissues. Food and nutrition, and biochemical individualities, do indeed alter mood, cognition, and behavior.(42)

The ability to easily and inexpensively measure and monitor amino acids, vitamins, and other analytes is likely to lead to new understanding and eventual modulation of dietary and personal neurochemistry.

Preliminary Studies

Five years ago (43) we became interested in the application of bioluminescence (44) as a means by which to analyze a wide range of important metabolites. We also became interested at that time in patient empowerment (45) and in-home based medicine (www.ee.cua.edu/~winters/HCTWorkshop). We felt strongly that if patients were going to be significantly involved in the management of their health, they were going to need tools by which to monitor and measure their physical and chemical states.

We now know that most diseases and other pathologies are complex – that biochemical reactions do not exist or operate in isolation. Every reaction is obviously dependent on many other reactions through the principles of reaction kinetics and equilibria. And yet we continue to look for "magic" single chemical parameter disease correlations. We study them and present those results to the general public in the form of lifestyle recommendations, never warning them that this is such a tiny part of the biochemical "elephant" that it may well be irrelevant to the overall system. We must have the tools to move beyond mono-parameter chemical paradigms. We need devices which can easily and inexpensively measure relevant biochemical parameters – to provide the information base to more fully understand and treat biochemical diseases.

Bioluminescence has the analytical advantage that it is generally at least 100 times more sensitive than common spectroscopic/colorimetric methods.(4) (46) Bioluminescence is light produced by biological compounds undergoing specific, enzyme catalyzed oxidation reactions. The most well known example is the firefly; however, other organisms employ similar reactions to produce light, e.g., bacteria, fish, and fungi. All bioluminescent reactions employ an enzyme called "luciferase" which facilitates the oxidation of an energetic substrate, called "luciferin", into an excited state which emits a photon – a chemiluminescent process which is called

bioluminescence because it requires enzymes. There are many different luciferases and luciferins; there are at least 30 different known bioluminescent reactions in nature.(46) There is a unique firefly luciferase and luciferin, a unique bacterial luciferase and luciferin, etc.

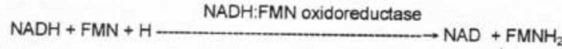
The approximately green bioluminescence of fireflies is based on the enzyme catalyzed oxidation of luciferin utilizing adenosine triphosphate (ATP) as a highly specific co-reactant. The blue bioluminescence of marine bacteria is closely coupled to a reduced nicotinamide adenine dinucleotide (NADH)-dependent enzyme reaction. ATP and NADH represent the "energy currencies" of metabolism - of all life forms on the planet.

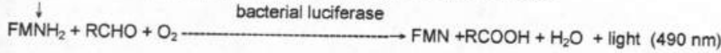
Thus, Mother Nature has literally given us two unique, ultra sensitive and highly specific reactions for the measurement and monitoring of ATP and of NADH (and NADPH, the phosphorylated form). The readout is photons, green-yellow in the case of the firefly reaction and blue for the bacterial reaction. The reactions are sensitive to ATP or NADH/NADPH over 5 orders of magnitude in concentration(4). Since most of biochemistry depends on ATP and/or NADH, practically all metabolic reactions can be monitored by bioluminescence via one or more enzyme linked reactions (see a biochemical or metabolic pathways chart or www.expasy.ch/cgi-bin/search-biochem-index).(47)

There is a large body of literature on the development of sensors for ATP- and NADH-dependent processes, using the firefly luciferase and bacterial luciferase enzyme reactions, respectively. For some of the analytes of interest, the bioluminescence is of sufficient intensity that the unaided eye can serve as the detection instrument,(43) although generally a photomultiplier tube (PMT) based luminometer is used. The intensity of bioluminescence is proportional to the concentration of the specific biochemical of interest in the sample.

Although bioluminescence analysis is well known and has been used regularly in research and in analytical and clinical laboratories, it has not been widely applied outside those specialty areas for several reasons:

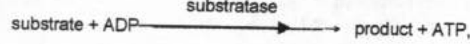
1) The exquisite sensitivity for very low ATP concentrations has encouraged the application of the technique to those problems where such sensitivity is indeed needed. Thus, it acquired the reputation of an ultra sensitive technique and has not been seriously considered for the measurement of analytes in the micromolar to millimolar range.
2) The luciferases and other reagents involved have developed a reputation of being somewhat labile, unstable, and difficult to utilize. Additionally, sources of various luciferases have until recently been expensive and often of questionable quality.
3) The nature of the bioluminescence reaction, and in particular its complex kinetics, made it necessary to develop rapid mixing techniques and to utilize an instrument capable of sensing a flash or short pulse of light. Application to trace concentrations required a highly sensitive, and therefore relatively expensive, luminometer. Thus, the technique evolved a reputation for requiring an expensive instrument, and a precise and somewhat sophisticated analysis protocol.
4) The wide spread application of the firefly luciferase reaction to the monitoring of very low concentrations of ATP released from bacterial and other cells in hygiene monitoring applications lead to the idea that the "cocktails" (the surfactants, detergents, and other agents required to disrupt cell membranes) denatured and inactivated the luciferase involved; therefore, such applications required a delicate balance, a careful optimization, and were often difficult to carry out in a reliable and reproducible manner.





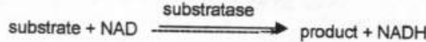
In both cases the intensity of the light produced is directly proportional to the reaction rate, which is proportional to the ATP or NADH concentration. Hence, sensors for ATP or NADH concentration. Several of the bacterial luciferase systems are also sensitive to NAD(P)H, which is also analytically useful.

A simple substrate-specific sensor is one in which the substrate-specific enzyme reaction produces ATP:

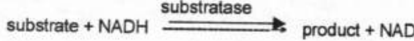


where "substratase" is the substrate—specific enzyme. A reaction which consumes ATP can also be used.

Reactions involving the production of NADH:



or the depletion of NADH:



can also be used.

The sensors can be designed so that the substrate—specific reaction is largely completed before the output is mixed with the appropriate bioluminescence reagents – we call this a heterogeneous or 2 step sensor. A significant advantage in the development of these biosensors is that the bioluminescence reactions are quite slow in comparison with the substrate-specific reactions, meaning that the entire assay can be conducted in a single step – we call this a homogeneous or single step sensor. This characteristic greatly simplifies sensor and Chip design and application, because the various biochemical reactions are occurring in the same volume simultaneously and competitively. The advantage of a homogeneous assay is that it involves fewer assay preparation and/or device design steps. We are, of course, aware of the pH optimum differences among enzymes, and of other issues. It is a matter of optimizing the set of competing reactions to provide a useful analytical output. This is now greatly facilitated by enzyme kinetic modeling and simulation (Task 2) and by enzyme engineering and expression (Task 3).

The sensors are designed for discrete samples. The concentration is measured using a simple disposable device. Both the device and the sample are then discarded. For medical and clinical purposes, the present sample of choice is blood, usually derived from a simple lancet-based fingertip, earlobe, or heal prick. Modern micro-lancets readily generate a 50 microliter droplet, adequate for the proposed multi-analyte ChemChip.

Our work to date was focused on establishing the practicality of the ATP and NADH sensors, and the feasibility of specific substrate sensors using the ATP and NADH platforms. We choose

Several years ago, sponsored by the Whitaker Foundation/National Science Foundation Program in Cost Reducing Health Care Technologies (CRHCT) (www.healthtechcost.med.utah.edu), we began a project to develop sensors for the monitoring and management of selected inborn errors of metabolism: phenylalanine in the case of phenylketonuria (PKU) and galactose in the case of galactosemia. We have also done preliminary work on sensors for creatine and creatinine.(48)

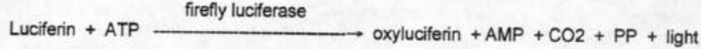
Although our original goal was the development of simple devices which would not require an instrument (instrument less biosensors), our interactions with the clinical community have taught us that they prefer an instrument be used in order to provide an objective number, to record data, to process and analyze data and establish trends, and to transfer that data and those trends to the health care provider. Having an instrument with which to read the signal produced from such sensors greatly expands their analytical capabilities. The remarkable sensitivity of bioluminescence, coupled with the sensitivity of a modern CCD-based luminometer, makes possible the measurement of submicromolar concentrations in small (less than one microliter) samples. Our interaction with the clinical research community also taught us that they have major needs for effective, inexpensive ways to measure multiple analytes for disease research and for treatment development (Task 1).

We have developed ATP and NADH detection platforms over the past several years. Our approach is based on the following considerations:

5) The biotechnology community now knows how to express, produce, and purify proteins via simple organism cultures and processes. Indeed, recombinant firefly and bacterial luciferases have been known for several decades now, and recombinant firefly luciferase is commercially available (www.promega.com). Highly robust, temperature stable luciferases and other enzymes are now produced via directed evolution methods.
6) The biotechnology and protein pharmaceutical industries have learned how to formulate, passivate, store, and reconstitute proteins and enzymes with considerable retention of activity.(49) We have addressed the instability of firefly and bacterial luciferase using our experience, understanding, and control of the denaturation of proteins at interfaces and in solution.(50)

3) A reaction which actually produces photons has many advantages: it does not have the problems associated with color perception or wavelength separation, as in the case of reflectance colorimetry; it does not require a light source, as in the case of fluorescence spectroscopy; and it does not require electrodes which can become contaminated, as in the case of much of analytical electrochemistry.

The ATP platform is based on the firefly luciferase reaction:



$$\text{Luciferin} + \text{ATP} \xrightarrow{\text{firefly luciferase}} \text{oxyluciferin} + \text{AMP} + CO_2 + \text{PP} + \text{light}$$

The NADH platform requires a two enzyme process:

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galactose as the prototype ATP-dependant analyte (using galactokinase) for application to galactosemia (51) (52), and phenylalanine as the prototype NADH sensor (via phenylalanine dehydrogenase), with PKU as the specific application.(53) (54) There are literally dozens of other specific dehydrogenase enzymes which will allow the measurement and monitoring of many other specific analytes. Indeed, one can analyze all common amino acids by this process.

With the close collaboration of Dr. R. Stewart, we have engineered and recombinantly (E. coli) produced a set of enzymes with which optimal and enhanced sensors can be developed:
Firefly luciferase (55)
Firefly luciferase with a BCCP (biotin—expressing) domain;
Firefly luciferase with a polyhistidine tail;
Bacterial oxidoreductase (Vibrio fischeri)
Bacterial oxidoreductase with the BCCP domain
Bacterial luciferase (Vibrio harveyi)
Bacterial luciferase with BCCP domain (56)
Galactokinase
Galactokinase with BCCP and polyHis (52)
Phenylalanine dehydrogenase (Bacillus badius)
Phenylalanine dehydrogenase with BCCP domain and polyHis tail

The polyHis tail permits simple, one step purification of the E coli—produced proteins. The BCCP domain permits specific binding to avidin or to streptavidin, two biotin-specific proteins. This permits ready immobilization of the enzymes to beads and/or other surfaces as desired, with little or no loss in activity. We have also initiated DNA shuffling (directed molecular evolution (57)) studies of the Lux (bacterial luciferase) gene, resulting in a bacterial luciferase with significantly enhanced thermal stability. The availability of such unique proteins enhances sensor development and application.

These examples are presented to demonstrate the power of the technology to be utilized in ChemChip development. With the functioning platforms for ATP and NADH analysis, and the experience and progress with galactose and creatinine (both ATP based) and phenylalanine (NADH based), it is now straight forward to apply a new enzyme "front end" (a new substratase) to impart the specificity needed for a particular analyte.

Luminometers are currently used for bioluminescent-based analytical measurements. 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 microfabricated sensor chips using imaging CCD detection. We have also shown that silver coated micro-wells have the ability to enhance light output at low sample volumes. Through careful design of microfluidic channels and light enhancing devices, such as silver coated surfaces, bioluminescence can be applied to ChemChip devices for accurate measurements of multiple metabolites from low sample volumes.

The ChemChip we propose would, literally, quantitatively "image" the metabolism associated with biochemical diseases. The image of a PKU patient's metabolism would be significantly different from that of a galactosemic patient, or a diabetic. The ability to measure – to image in chemical terms – metabolism would be a great asset to research as well as to clinical and preventative medicine.

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A major benefit of biosensor chips will be reduced cost. Using the ubiquitous microprocessor as a metaphor, ChemChips could be manufactured by the millions, thus greatly reducing the cost per chip. A physician or patient need not use all the data generated, but merely focus on the several channels of immediate clinical need and interest. The other channels could simply be ignored by the custom programmable device, much as is now the case with microprocessors used for specific, embedded, applications. Thus the economic problems associated with the development of a home assay for PKU, or for galactosemia, or any other low population condition, are minimized by simply producing one Chip which is useful for many, many applications, and will be invaluable to clinical research. Generally many of the analytes needed for one disease are also important and relevant to other diseases, due to the interrelationship and highly interconnected nature of metabolism.

Economics

Our ongoing Whitaker/NSF Grant (which terminates 12/2000 and cannot be renewed) has included a significant economic analysis component. Drs. Robert Huefner and Norman Waitzman, and their colleagues, have worked closely with us over the past 3 1/2 years to study and evaluate the cost benefit issues associated with PKU. They have developed a model and methodology to evaluate the cost effectiveness of potential new medical technologies. This work is now being prepared for publication in a series of papers for both the Bioengineering and health economics communities.

Much of the work was discussed at the February, 2000 meeting of the the American Association for the Advancement of Science (AAAS) in Washington D.C. Our group, and particularly Steven Kern, Edward Clark, and Norman Waitzman, all participants in this proposal, organized two symposia at that major national meeting. The first provided an overview of the issues pertaining to economic and policy influences on healthcare technology development. The second symposium treated the specific case of the development of technology for children's health. These symposia helped raise awareness of these issues in both the scientific and policy communities and provided a forum for communicating these issues to a broad audience.

The discussion will be continued during the Feb. 2001 AAAS meeting in San Francisco, in a symposium organized by Kern and Clark (Proposal # 6081) titled:
Screening and Management of Inborn Diseases: Trouble in the Postgenome Days

Proposed Participants include:
Edward R.B. McCabe, M.D., Ph.D. Executive Chair, Department of Pediatrics and Physician-in-Chief, Mattel Children's Hospital at University of California Los Angeles;

David Millington, Ph.D., Professor of Pediatrics, Duke University Newborn Screening Program Duke University School of Medicine, Durham North Carolina;

Dallas or Tera Mize, Founders, TYLER FOR LIFE FOUNDATION, INC. Douglasville, GA 30134-4023 -- A parent's perspective on neonatal screening and management for inborn diseases;

Norman Waitzman, Ph.D. Associate Professor of Economics, University of Utah, Salt Lake City, Utah 84112;

Philip Lee, M.D. Institute for Health Policy Studies, San Francisco, California; former Assistant Secretary of Health and Human Services.

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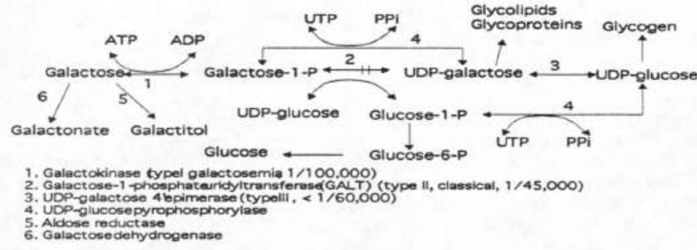

Figure 4 Galactose metabolism

It is possible that galactitol and/or galactonate are responsible for the chronic, insidious problems of galactosemics; it is also possible that their problems may be due to abnormal production and/or synthesis of galacto-proteins or galacto-lipids. The issue is far from clear. The research community needs means to measure the four key analytes and three key enzymes involved in galactose metabolism – a complete "panel" for galactosemia.

We will also consider transplantation, the major therapy for patients with end-stage organ failure (kidney, liver, heart, lung, and pancreatic β-cell). Renal transplantation alone accounts for more than 65% of the total solid organ transplantation in the United States. The availability of a home sensor for creatinine and urea will permit long-term renal transplant monitoring in the home environment. Such assays will improve convenience and life quality, and minimize renal transplant failures that now go undetected and untreated due to infrequent monitoring. We will continue and expand our discussions with the clinical and research transplant community and assess their sensing and monitoring needs for measures of organ function and dysfunction.

Although we do not address TDM via immunoassay or HPLC in this project, we may address it via our enzyme bioluminescence approach. Two major immunosuppressants, cyclosporin and tacrolimus, are derived from a fungus; the synthetic and metabolic pathways of these fungal biochemicals are being elucidated, meaning that specific enzymes for their synthesis and biochemical processing are becoming available. All we need is a substratase which we can couple to ATP or NADH. Thus it is likely that we will have reactions and enzymes to permit the development of ChemChip channels for these and related drugs for the concentration ranges now in clinical use.

The ketogenic diet apparently causes a switch in the energy producing metabolic pathways in the body. The absence of glucose as a fuel and the presence of abundant fatty acids induce nonglycolytic pathways, normally present only in starvation situations. (24)Our discussions with the clinical epilepsy community and a review of the available literature suggests that the field needs far more chemical information. In order to understand the bioenergetic implications of epilepsy and of the ketogenic diet, it is clear that measures of ATP, ADP, glucose, creatine, lactate, pyruvate, hydroxybutyrate, pH, carnitine, and possibly adenosine would be extremely informative. The disease, the diet, and even drug treatment can lead to serious side effects which can be alleviated and hopefully understood by a measurement of ammonia, phosphate, and the water-soluble vitamins. Vitamins B, C, and D, as well as folate, could also be measured. It is also recognized that epilepsy does lead to other amino acid concentration abnormalities. One hypothesis as to the nature of epilepsy and the possible role of the ketogenic diet is that

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Although we do not have the resources in this proposed project to continue major economic and health policy studies, we certainly do want that perspective and such discussions available for our ongoing studies. Therefore we have included both professors Waitzman and Huefner, as well as Philip Lee, Brent James, and Owen Ash, as members of the National Advisory Board for this BRP project (they are all participants on our current National Advisory Board and Clinical Advisory Team).

Research Design and Methods

Task 1: Disease Management and Clinical Research Needs

The function of Task 1 is to obtain input from the clinical and clinical research community on the biochemistry, nutrition and diet, and therapeutic drug aspects of the diseases and clinical conditions addressed in this proposal. We ask the clinical research community what chemical information they need for disease management and what tools they need for clinical research. These inputs will be considered and evaluated in Task 2.

We will initiate the activities by continuing our focus on selected inborn errors of metabolism, particularly PKU and Galactosemia. There is growing recognition of the need to better monitor and manage inborn metabolic diseases. Studies of tightly monitored and regulated Type I Diabetes have demonstrated both the health and economic benefits of frequent monitoring and aggressive disease management. The phenylketonuria (PKU) community is also beginning to recognize such benefits. Although these are relatively rare diseases, the patients and their health-care providers have specific analytical chemistry needs which can be readily addressed.

A galactose sensor for Galactosemia and a phenylalanine sensor for PKU serve as nearly ideal model systems for the application of the ATP and NADH bioluminescence analysis platforms, respectively. These two diseases also allow us to dramatically increase our analytical repertoire: PKU management requires more information than just phenylalanine; it also requires tyrosine, leucine, isoleucine, valine, and methionine.(58) Due to the role of phenylalanine and tyrosine as chemical precursors for dopa, dopamine, and epinephrine, the Task 1 clinical discussions may well result in strong recommendations for means to measure those important neurochemicals.(42)

Although PKU patients are routinely managed by control of phenylalanine intake and can lead relatively normal and productive lives, this is, unfortunately, not the case for Galactosemia. See Figure 4. It is not possible to control the galactose and G-1-P concentrations by diet alone, since galactose is synthesized and produced by a variety of metabolic reactions. Even if galactose is rigidly controlled via dietary lactose restriction it still accumulates in the blood and tissues due to the presence of galactose in many foods, including fruits and vegetables. Also, galactose is an endogenous component as it is a key component of galacto-proteins and galacto-lipids. Galactose can apparently be incorporated in these compounds via the glucose-1-P and UDP-galactose pathways. The natural turnover and recycling of galacto-proteins and related compounds results in the endogenous production of galactose. The resultant elevated galactose and Gal-1-P levels promote the production of galactitol and galactonate, resulting in their abnormally high levels. Galactose can be reduced to galactitol via aldose reductase. Galactitol is apparently responsible for the cataracts characteristic of galactosemia. Galactose may also be oxidized to galactonate by galactose oxidase. The galactonate can then be metabolized to xylulose and carbon dioxide.

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the change in metabolic pathways may result in altered levels of amino acid transmitters, particularly glutamate and aspartate.

Task 2: Modeling and Simulation of Biochemical Networks

Modeling and simulation of biochemical networks is helpful in understanding metabolism and its role in disease. One can predict how altering the concentration of one or more analytes affects the concentration of other metabolites. This not only offers the advantage of being able to provide a more complete metabolic profile of a patient, it can be used as a research tool to better understand the widespread implications of specific diseases. In the case of impaired glucose metabolism, for example, several metabolic pathways, including lipolysis, glycolysis, and gluconeogenesis, are affected. It is possible to predict how changes in glucose levels affect the formation of metabolites such as ketone bodies, pyruvate, and lactate. Ultimately, this can improve disease management and minimize serious complications.

Models of biochemical systems can be descriptive, explanatory, or predictive. The qualitative and quantitative relationships between states of the biochemical systems can be estimated with descriptive and explanatory models, while predictive models can be used to estimate the behavior of the system under novel conditions.

Although data sufficient for a complete quantitative description of most biochemical systems related to possible metabolic pathologies may not be currently available, many useful qualitative observations of biochemical system can be obtainable though modeling and simulation.(3) The impact of the individual components (metabolites, enzymes, reaction environment, etc.) of the biochemical system can be qualitatively ascertained. Therefore, the behavior of parameters or components of the biochemical systems which may be critical can be estimated even with incomplete quantitative data.(59) Furthermore, when the model only seeks to find the limits of behavior rather than provide quantitative predictions of the behavior, the completeness of quantitative data becomes less important. Additionally, qualitative analysis of a biochemical system model may provide insight in the behavior of the system not obvious in single quantitative models. As more quantitative data becomes available the models can be adapted appropriately, such as by reducing their limits or bounds, with the ultimate goal being a quantitatively predictive model of a particular metabolic pathology. This "soft modeling" technique has been used in metabolic system modeling by Mavrovouniotis(3), where the thermodynamic and kinetic bounds of the system were examined.

The relatively new fields of metabolic control analysis, metabolic engineering, and metabolic mapping are dealing with the complexity inherent in thousands of biochemical reactions which are all interdependent.(60) (61) Given the importance of metabolism it is surprising that there has been relatively little effort in attempting to analyze and measure key metabolites. Metabolic pathway analysis allows the definition of the biochemical state space which defines the necessary input and output conditions to appropriately monitor the formation of a specific metabolite of interest.(62-64) This approach uses linear algebraic methods to determine the biochemical eigenvectors relevant to the pathway under consideration. When combined with the discussions and input from Task 1, we will select the analytes for sensor development. Using pathway analysis may also allow for the identification of biochemical surrogates that are easier to measure than the analyte of primary interest yet reflect in a quantitative relationship to the primary analyte.(62, 63)

Since all biochemical systems are generally complex and non-linear in nature, non-lineai modeling techniques and tools will be used to characterize the reactions within the ider

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pathway space. Two basic approaches to modeling non-linear biochemical systems will be used in this task: phenomenological (canonical formulation (65)and/or Fliess-Volterra formulations) and extended enzyme kinetics. (Table I)

The phenomenological approach can be viewed as top down from a simple input /output model to an increasingly complex model with the addition of extra parameters. The phenomenological approaches have the advantage of being easier to set-up and the number of parameters can be controlled. The canonical or Savageau formulation is based on power laws or polynomials to describe the non-linearity of the system, while the Fliess-Volterra formulations are based on standard non-linear control theory.

The extended enzyme kinetics approach is a bottom up approach, starting with the most complex and accurate model, which is increasingly simplified in order to manipulate and evaluate the biochemical system. Extended enzyme kinetics refers to using standard enzyme kinetic formulations, but extended in the sense that the standard assumptions generally applied to linearize the system are not used. The extended enzyme kinetic approach more accurately describes the biochemical system, but is computationally more intensive.

Singular perturbation theory, normal forms, and center manifolds are analytical techniques that can be applied to the non-linear systems of ordinary differential equations for each of the approaches. The techniques are implemented through use of symbolic manipulators such as Maple or Mathematica to provide simplification by isolating the heart of the dynamics of the non-linear system.

Robust numerical ordinary differential equation (ODE) solvers will also be used to provide solutions to the non-linear system.

The models will be used to help assess which metabolites are critical to the dynamics of the biochemical system (origin of the pathology) and would therefore be the most important to measure. The ultimate goal of an accurate model is the prediction and then minimization of detrimental swings in critical metabolite concentrations. In other words, the treatment of certain pathologies can be changed from a costly reaction-to-symptoms-based (which may be too little too late) to inexpensive prevention-based.

A schematic view of the modeling process is in Fig.5 (65) (66) (64, 67) The "dissected" metabolic pathways are studied with the key metabolites (what is now considered as most important), then the more complex model using "peripheral" or additional reactions, which likely leads to the realization that the process is more complex than originally thought (additional key metabolite). Finally, the model can be extended and virtual experiments performed by assuming particular conditions or pathologies.

An initial application we will pursue is galactosemia. Consider Figure 4 in Task 1 (above), the reactions relevant to galactose metabolism. The 3 different galactosemias, each based on a single enzyme deficiency, were noted; 6 enzyme reactions are involved. Table III shows how the 4 key metabolites and the 3 enzyme activities are altered in the 3 different galactosemias. If we had a seven channel sensor, we could measure all seven analytes, presenting the data (Task 5) in a radar plot configured to enhance the recognizability of the three galactosemias (Figure 6).

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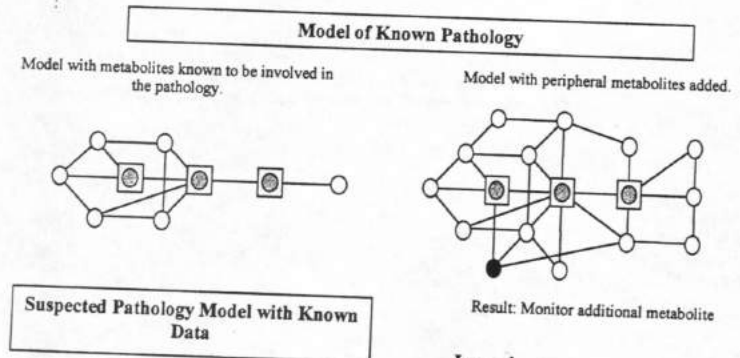

Fig 5 Graphical Representation of Modeling Task Goals

Table III 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.

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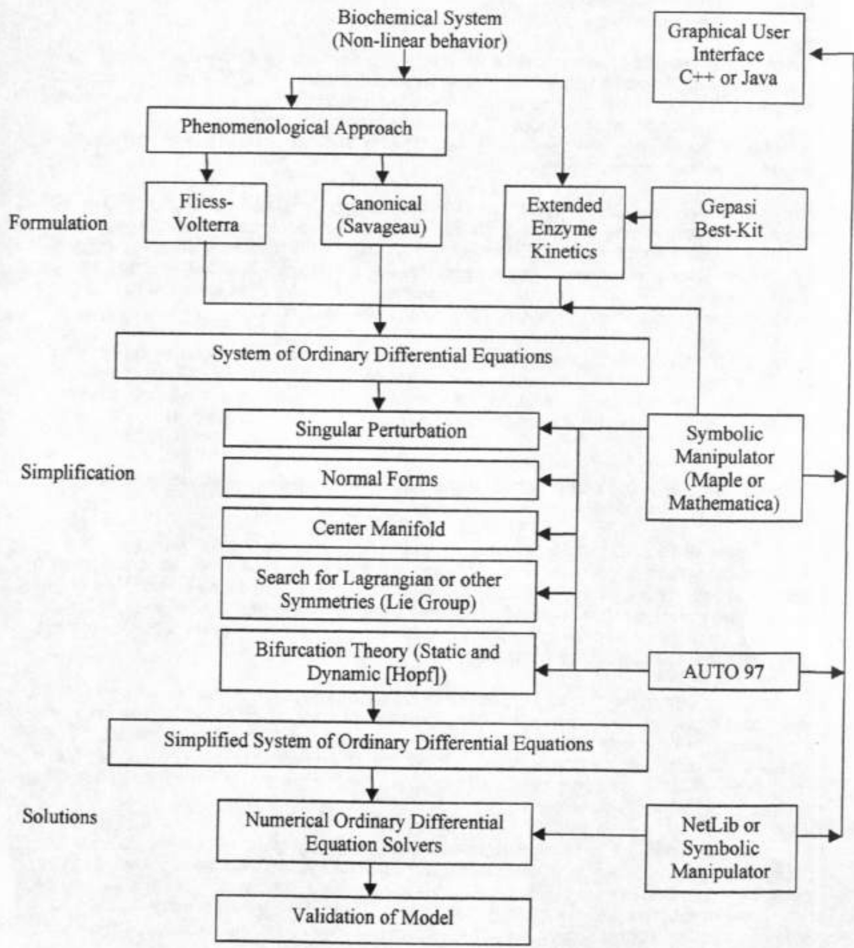
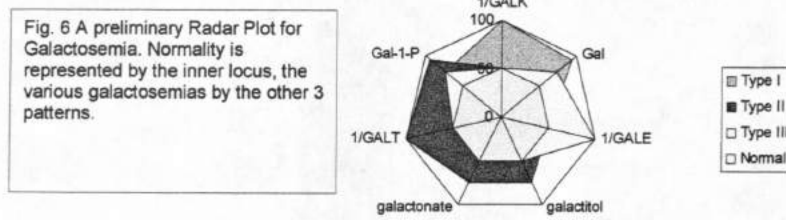
Table I: Modeling Tools



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 "substratase" 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) (68)

Fig. 6 A preliminary Radar Plot for Galactosemia. Normality is represented by the inner locus, the various galactosemias by the other 3 patterns.



A further objective of the modeling task is to evaluate the metabolic reactions available with which to couple to the luciferase assays in terms of optimum performance for a functional analyte assay. The most important performance parameter is an analytical range similar to the physiologically significant range. This work will integrate the modeling with the experimental task of developing functional metabolic assays (Task 3). The goal is to identify the key parameter values needed for a successful assay, thereby minimizing the number of laboratory experiments required

We will model all our enzyme-based analytical reactions, initially for feasibility and then for optimization. Sensing performance, including signal output, signal pattern, and resolution, can be enhanced by control of buffer type, concentration, pH, temperature, and enzyme amount (activity). The results can also be optimized by assay volume, enzyme status (immobilized or free), and enzyme type or source (kinetic parameters).

An example of the initial stages of the analyte reaction development process is the creatine example.(48) We chose to use an ATP depletion assay based on a creatine kinase (which phosphorylates creatine). First, we model the ATP detection reaction, using the multistep kinetic model and constants of Gandelman, et al. (69) All computer modeling was performed using Gepasi, a program for the simulation and optimization of biochemical reactions.(66) We use an initial luciferin (LH_2) concentration of 30 μM, luciferase concentration of 0.7 μM, and ATP concentration ranging from 0.3 μM to 3.3 mM. The creatine reaction was modeled to determine feasibility and optimal conditions. The maximum velocity and Michaelis-Menten constants used in the model for the CK reaction were for rabbit muscle creatine kinase. For the coupled reaction, the luciferin concentration was between 0.01 and 1 mM, the luciferase concentration between 3×10^{-4} and 3×10^{-3} mM, and the ATP concentration between 0.1 μM and 1 mM.

To validate the computer model, experiments were performed using a Turner 20/20 luminometer to measure the light output as a function of time. The difference between the ATP

consumed by the firefly luciferase reaction alone and the ATP consumed by the coupled reactions is proportional to substrate concentration. This difference is obtained from the light output of the creatine kinase—luciferase homogeneous reaction (one channel) and the luciferase reaction alone (a second channel). A set of optimum conditions was estimated from the simulation and then a preliminary set of experiments were conducted. The general conclusion was that such a sensor is indeed feasible and merits development. Since the creatinine sensor utilizes a creatine sensing channel, this work also provides a creatine sensor, an important metabolite with its own clinical analysis needs and applications.

The design parameters for a biosensor are based on the required resolution and sensitivity of the assay. For example, a biosensor that measures creatine must be capable of detecting creatine concentrations between 0 and 300 μM with a sensitivity of 10 μM and a precision of $\pm$ 5%. To achieve these results, both the reaction kinetics and instrumentation limitations must be considered.

We have also developed a preliminary model of the phenylalanine sensor reactions which matched experimental results reasonably well.(54) The model can now be extended and used to optimize sensor design. The model allows the designer to adjust enzyme concentrations, ratios, enzyme activity, sample volume to control bioluminescence peak shape, time course, plateau characteristics, and response to Phe concentration.

Task 3: Analytes and Analytical Methods

The four subtasks are:

- Enzyme Assays
- Enzyme Production and Characterization
- Reagent and Sensor Stability, and
- Calibration and Validation.

Enzyme Assays-- We can, in principle, produce an analytical channel for any substrate or enzyme involved in a specific reaction which produces or consumes ATP or NADH (or NADPH). The substrate—specific enzyme reaction need not directly involve ATP or NADH. One approach to a creatinine sensor, for example, is to use a creatinine to creatine reaction (creatininase), a creatine phosphorylation reaction (creatine kinase), and the firefly luciferase reaction. The creatinine "sensor" is basically a creatine sensor (discussed above) with a creatininase "front end". As long as we can couple the substrate/substratase reaction to ATP or NADH, we can make a sensor.

The reactions below demonstrate how a dehydrogenase reaction is used in a sensor for lactate using lactate dehydrogenase (LDH).(70) In the reactions below, we can replace the lactate substrate with an amino acid of interest, replace LDH with the specific dehydrogenase for that amino acid, and one has a first attempt at a sensor for that specific amino acid. We have optimized this approach for phenylalanine.(53, 54, 71)

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As an example, consider the preparation and production of biotinylated phenylalanine dehydrogenase for coimmobilization with biotinylated bacterial luciferase and oxidoreductase using biotin-avidin interactions. This coimmobilized three enzyme system can measure phenylalanine. Biotinylated phenylalanine dehydrogenase (PDH) was produced in E. coli by genetic fusion with the biotin carboxy carrier protein (BCCP). The PDH gene was PCR-amplified from bacteria strain Bacillus badius. BCCP gene was also PCR-amplified from a plasmid BCCP-lux plasmid constructed in our lab. The PCR products of PDH and BCCP were digested with NdeI and BamHI, and XhoI and BamHI, respectively. The PCR fragments were ligated simultaneously into pET-26b digested with NdeI and XhoI. A His-tag and the BCCP domain were introduced onto the end of N-terminal side of PDH as a result of the subcloning. The BCCP-PDH was expressed in E. coli strain BL21(DE3). The 500-ml culture containing kanamycin and biotin was grown at 37 C until OD600=0.9 and then induced at 30 C for 4 hours with IPTG. The His-tagged BCCP-PDH was purified using a Ni-NTA agarose column. The biotinylated PDH was expressed well and showed almost the same activity as a wild type PDH.

Reagent and Sensor Stability-- Enzyme stability is key to the development of biosensors. It is important to select enzymes which are inherently robust and retain activity after lyophilization, deposition, and rehydration.

Lyophilization stabilizes enzymes for long-term storage by reducing both mechanical and chemical degradation. Mechanical or physical degradation includes aggregation or precipitation, while chemical degradation includes oxidation, deamidation, hydrolysis, etc. Although lyophilization generally increases the long-storage stability of protein, the processes of lyophilization (freezing and drying) can also denature the enzyme. However, with appropriate stabilizing excipients and preservatives, the denaturation often observed during the processes of lyophilization can be minimized.

We have studied the stabilization of creatine kinase, a particularly delicate enzyme, as a model. Process variables which must be optimized include
the initial concentration of enzyme, buffers that exhibit minimal changes in pH with freezing and drying, rate of freezing (should be slow), and various additives. The glass transition temperature of the amorphous phase of the lyophilized enzyme should exceed the planned storage temperature. Additionally since water will plasticize the amorphous phase, low water content is necessary to insure glass transition temperatures are greater than storage temperatures. The glass transition temperature of the lyophilized enzyme can be increased by disaccharides and polymers (used in combination with disaccharides).(49; 75) Disaccharides such as sucrose and trehalose are especially good at stabilizing the enzyme during freezing and and dehydration. The sugar to protein weight ratio should at least 1 to 1, although stability can be further increased with greater sugar (5 to 1). Reducing sugars such as glucose, lactose, maltose or maltodextrins should 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. Furthermore, surfactants can be used to inhibit aggregation at very low concentrations, such as less that 0.5% per volume.

Since firefly luciferase is sensitive to oxidation due to sulfhydryl groups critical to its activity, antioxidants such as dithiotheitol and glutathione are used during lyophilization and subsequent storage. Furthermore, since firefly luciferase is very surface sensitive, bovine serum albumin is used for surface passivation. Polyethylene oxide (PEO)-based polymers and surfactants are also effective. Since moisture is a major culprit for both physical and chemical denaturation, lyophilized enzyme should be stored with appropriate desiccants to avoid water absorption of moisture. Storage must be in the dark to minimize photooxidation.

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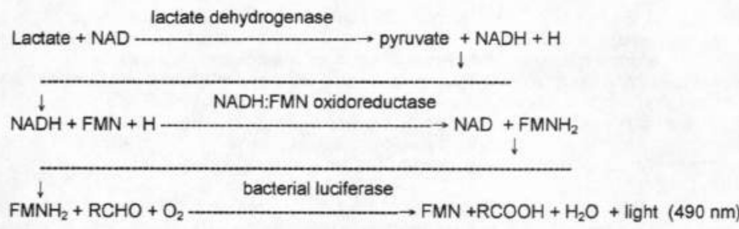

The equations represent an NADH/$FMNH_2$ production reaction where the added reagents are nicotinamide adenine dinucleotide (NAD), flavin mononucleotide (FMN), long chain aldehyde (RCHO, i.e., dodecanal), and the three enzymes noted. The intensity of bioluminescence (λ_{MAX} = 490 nm) is directly proportional to the substrate concentration in the sample (lactate in this case).

Note that the dehydrogenase reaction produces NADH which is "consumed" by the oxidoreductase reaction, producing FMNH2 which is then "consumed" by the luciferase reaction (as well as by direct oxidation, which we have also modeled and studied ()). Each reaction is driven to completion by the consumption of its product. This makes for an efficient, straight forward sensor.

Each sensor requires an analyte-specific enzyme (LDH in the above example). Although many such enzymes are available from commercial sources, it is often convenient and even necessary to produce them by recombinant means, as we noted earlier.

Enzyme Production and Characterization-- Our work to date has been performed in the lab and under the supervision of Dr. R. Stewart.(53, 54, 56, 71) (51, 52, 55, 68, 72-74) The needs in this project exceed the capabilities of Dr. Stewart's lab, thus we are establishing a preparation and small production facility within our new biosensors laboratory. We are fortunate that Dr. J. Janatova will join our group to set up and run this lab. She is a protein biochemist and Assoc. Research Professor of Bioengineering and formerly directed a Bioprocessing Core Facility (BCF). Her experience in the production, purification, and characterization of a variety of proteins will be very helpful.

The enzymes required for sensing will be produced recombinantly in high purity and yield. Recombinant techniques allow us to add other functional domains to the target protein. We have successfully expressed firefly luciferase, bacterial luciferase, oxidoreductase, and galactokinase in either pET vectors (from Novagen) or pRSET vectors (from Invitrogen). The recombinant enzymes are fused with a 6-Histidine tag carried in the expression vectors at either the N- or C-terminus. The histidine residues chelate Ni^{++} ions with high affinity (two histidines per Ni^{++}, $Ka=10^{13}M^{-1}$). The recombinant proteins are thus purified using immobilized-Ni^{++} matrix using a simple elution process.

We are now working on recombinant, engineered PDH, expecting to produce material suitable for rapid, inexpensive production and purification. We will continue to work on PDH and various modified PDH, looking particularly to enhanced thermal and storage stability.

Fortunately, the protein pharmaceuticals community has been very sucessful at deriving formulations and processes to maintain enzyme activity under dry storage conditions. Various preservation and stabilization cocktails are well known and widely used.(49) 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) 10 mM $MgSO_4$, (5) 1 mg/ml of bovine serum albumin, (6) 1 wt% sucrose, and (7) 1 wt% Dextran T-40. The actual bioluminescent molecules (ATP, FMN, bacterial and firefly luciferase, Oxidoreductase, etc.) are added to the preservative reagents, mixed thoroughly and added to each channel of the pre-chilled biosensor. The biosensor and reagents are rapidly frozen to -70°C 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 lyopilization proceeds for an additional 24 hours at +30°C and < 100 mTorr of pressure. Air 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 bioluminescent activity for a minimum of six months.

Reagents necessary for the assays (especially luciferin and adenosine triphosphate) benefit from the same preservatives that protect firefly luciferase during lyophilization and storage. Luciferin is very light sensitive and must be stored in the dark.

Enzymes derived from thermophilic organisms are often inherently robust and very applicable, and can often be expressed in E coli or other production organisms.

We are now applying directed enzyme evolution (often called DNA shuffling)(57) to modify enzyme properties, including activity and stability. In contrast to traditional mutation methods, which require knowing the relationship between the sequence, structure, and function of proteins, DNA shuffling improves or changes enzyme properties by selecting and accumulating positive mutations. DNA shuffling is a technique for in vitro recombination of pools of homologous genes. The pool of genes is fragmented into random size pieces, and the PCR reassembly of full-length genes from the fragments via self-priming yields crossovers due to PCR template switching. During reassemby, point mutations are introduced at a very low rate. Coupled with appropriate selection or screening, this homologous recombination process is the most efficient known process for combining positive mutations and simultaneously removing negative mutations from the sequence pool. Many groups have improved enzyme thermal stability without loss of activity using DNA shuffling. Firefly luciferases with greatly enhanced thermal stability have been produced by the Promega Corp. group and should be commercially available soon.

DNA shuffling also allows us to modify the pH optimum, the Michaelis constant (K_m), and other reaction characteristics which are important for linearity, sensitivity, and range of the sensors. A K_m value for some substrates is sometimes lower than the normal concentration range in the sample. We may be able to adjust the K_m value and therefore the range by DNA shuffling. Finally, we can even change the substrate specificity of each enzyme, permitting assays which would otherwise not be possible.

As an example, it would simplify our work if bacterial luciferase had better thermal and storage stability. As DNA shuffling studies of bacterial luciferase were not available, we undertook a preliminary study. Lux gene (V. harveyi) was PCR-amplified from the plasmid constructed

previously in our lab. The PCR product was fragmented randomly by DNase I. The fragmented pieces between 50 to 300 base pairs were reassembled by PCR using a Taq polymerase. The reassembled full-length genes were amplified by PCR using proof reading polymerase (Dynazyme) and primers having a digesting site. This PCR product was digested with NdeI and XhoI and inserted into pET-28c digested with the same sites. A His-tag was introduced onto the end of N-terminal side of luciferase as a result of the subcloning. The lux gene libraries were expressed in E. coli strain NovaBlue(DE3) and screened with dodecanal. Only 20 colonies of 2300 colonies produced light. Five colonies showed better thermal stability in vivo. Modified luciferases from the 5 colonies were purified using a Ni-NTA spin column, and assayed at room temperature after exposure to temperatures of 20 C, 30 C, 40 C, 50 C, and 60 C, for 30 min. At 30 C, the modified luciferase showed 80% activity of the original activity while the wild type showed 40% activity. At 40 C, the modified luciferase showed 50% activity but the wild type luciferase showed 20% activity. Above 50 C, both luciferases showed almost zero activity. The specific activity of both luciferases at room temperature was the same. We have, therefore, significantly improved the thermal stability of bacterial luciferase by DNA shuffling. We plan to produce this bacterial luciferase in our new laboratory and use it for the NADH-based sensors.

Aldehyde Chemistry: The bacterial bioluminescence reaction requires an alkyl aldehyde reactant. Although our present assay formulations work well, the aldehyde has some volatility and odor. Therefore, we propose to produce a hydrophilic, low vapor pressure alkyl aldehyde . As aldehydes are intermediate in oxidation state between alcohols and carboxylic acids, a synthesis from alcohol or carboxylic acid precursors is not trivial. We will use reactive monofunctional polyethylene glycols (PEG), initially 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 result in bioluminescence, while exhibiting low volatility. In fact, it is likely that this aldehyde will have significantly greater activity that the conventionally used form.

We will perform the synthesis, purify and characterize the product, and evaluate its efficacy in the bioluminescence reaction. If it works as well as we expect, then we will further evaluate its stability and related properties.

Immobilization: The interaction between histidine and Ni can also be used for immobilization. In biosensor applications, enzymes are often immobilized on a solid support to prevent diffusion (into the sample solution) and minimize interference with other channels of the sensor. The performance of the sensor can be adjusted by changing the immobilized enzyme amount. Recombinant enzymes with BCCP domains can be immobilized through this interaction with high affinity ($Ka=10^{15}$ M^{-1}). There are a variety of solid matrices which can be used in immobilization.

Coimmobilization of sequentially operating enzymes improves total reaction efficiency, leading to higher sensitivity.(56) A coupled assay with coimmobilized luciferase and flavin reductase has the advantage that the $FMNH_2$ produced from the flavin reductase can be used more effectively for the luciferase reaction, reducing its autooxidation . Traditional immobilization methods use chemically conjugated enzymes on solid materials, resulting in low immobilization efficiency and low and inconsistent enzyme activity, due to nonspecific immobilization and surface-induced activity loss. We have applied the biotin-avidin system for protein immobilization, due to its high

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- Placement preparation and long-term stabilization of multiple enzymes in analyte specific chambers for repeatable measurements;
- Tests with blood (filtering and passivation against protein adsorption and clogging);
- Quality assurance features to test accuracy and stability of enzymes

Micromachining is a rapid technique to build ChemChip prototypes of various shapes and efficiently test the analytical channels for maximal light output, sample loading, and integration with a CCD. In order to take advantage of the light integration according to the Beer-Lambert Law, bioluminescent reaction channels will be created in various sizes ranging up to 2-cm long. Therefore, the initial design will look like Figure 7, with long channels to take advantage of the light integration properties. Many ChemChips with varying analyte channel lengths, widths and depths will be anisotropically etched into silicon wafers or molded into poly(dimethylsiloxane) (PDMS) (76) for rapid, inexpensive prototyping. Both of these methods are easily available in the HEDCO micro-machining facility at the University of Utah and via Dr. Frazier at Georgia Tech. The PDMS methods of fabrication are much less expensive that the etching of silicon and versatile in the possible channel shapes and surface modification. However, silicon wafer etching will also be used for prototyping due the ease of integrating electrochemical sensors within the chip as the ChemChip becomes more progressive to include other disease specific analytes.

Both methods are efficient enough to create multiple ChemChip designs on one wafer or substrate for characterizing the efficiency of various ChemChip shapes.
Initially, each prototype ChemChip will be cut to expose the ends of the analytical channels. Plastic wafers will be glued onto the back of each chip for support. Glass cover slides will be glued onto the silicon chips in order to observe capillary action and sample loading during the bioluminescent experiments.. Anionic bonding and pressure sensitive adhesives will also be used for bonding the glass to the silicon ChemChips. Glass can also be sealed to the PDMS ChemChips by plasma discharge oxidation of the PDMS substrates.

1-mM ATP/firefly luciferase/luciferin solutions will be placed in the sample reservoir and carried through the channels via capillary action. The light intensity will be observed at the ends of the analytical channels using a sensitive linear CCD (Figure 7). The light will be recorded at 1-10 Hz for 1 to 3 minutes (from the moment the ATP is added to the solution) and recorded as CCD counts. Data will be analyzed using MATLAB® where the light peaks, time, integrated light, and steady state values of the CCD counts will be compared for the different channel shapes. The CCD counts in the dark fields surrounding the channels will be digitally subtracted from the counts recorded from each channel in order to account for any scatter and background light. The channel shapes that produce the most efficient light intensity per sample volume will be determined by comparing intensity/channel volume with the different channel lengths, widths and depths for both silicon and PDMS ChemChips. These experiments will be repeated with silver coated ChemChips to determine if light output in long narrow channels is enhanced.

There is expected difficulty in coupling the open-ended channels to the CCD system. A thin rubber gasket with holes for the light channels can create a small space between the end of the channels and the CCD so that the bioluminescent fluid doesn't contaminate or touch the CCD array. This space will allow for gas to escape the channels as the sample fluid fills by capillarity, thus eliminating the need for a gas permeable, transparent membrane to cover the ends of the channels. The use of a linear CCD will also eliminate the need for wide angle lenses, waveguides and other optical covering devices should not be needed to couple the ChemChip to the CCD during measurements because the ends of the analyte channels should fill the entire

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affinity (K_a=10^{15}/M) and stability. We have produced V. harveyi luciferase and FRase I biotinylated in vivo, immobilized the proteins on avidin-conjugated beads, and used the enzyme beads to assay NADH.(56) Briefly, "the coimmobilized enzymes had eight times higher bioluminescence activity than the free enzymes at low enzyme concentration and high NADH concentration. In addition, the immobilized enzymes were more stable than the free enzymes. This immobilization method is also useful to control enzyme orientation, which could increase the efficiency of sequentially operating enzymes like the oxidoreductase-luciferase system."

Calibration and Validation-- The biosensors will first be tested in simple buffer solutions, followed by more comprehensive testing in commercially available blood certified free of HIV and hepatitis antigens. Internal standards will be added for calibration purposes. The accuracy, linearity and precision of the assays will be determined. Variations between various sensors will also be determined. Our results will be referenced against standard spectrofluorometric assays. 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 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.

Task 4: The General ChemChip

The design of the ChemChip will consist of many parallel channels specific for individual analytes. One of the significant advantages of ChemChips is that they will be designed to be mass produced, thus significantly reducing the cost of performing simple, quantitative, direct analyses of a wide range of carbohydrates, amino acids, vitamins, and secondary metabolites. A physician or patient need not use all the data generated, but merely focus on the channels of immediate clinical need and interest. The other channels could simply be ignored by the custom programmable device or stored for future reference.

Another significant advantage of the Chip is reduced sample volume. Using only about 50 microliters of blood or urine, up to 40 simultaneous, specific analyses could be performed, using only 1 microliter of sample per analysis channel. Such low volume is possible due to the great sensitivity of the bioluminescent approach to enzyme-based substrate analysis.

Our initial design (Fig. 7) will have a set of microchannels branching from a reservoir, from which they will draw the sample fluid via capillarity to an array of microreaction chambers (MRC). Each MRC would have an individual set of reagents (from Task 3) which would initiate different reactions coupled with bioluminescence. The luminescence over time from each well would be stoichiometrically and dynamically proportional to a specific metabolite according to each unique set of reactions.

The specific goals of this project focus on the development, design and microfabrication procedures of the ChemChip for:

- Maximal light output for each analytical channel with minimal, signal scatter or interference;
- Effective integration of the ChemChip with a CCD and a data processing computer for simple instrumentation;
- Analytical channel features that promote efficient loading (or filling) and mixing liquid samples into each of the analytical chambers without bubbles or clogging while maintaining precise volume control;

view of the linear CCD for maximum spatial and light intensity resolution. Current linear CCDs range 2,000-10,000 pixels by 1-10 pixels (30-70 mm by 0.007-0.3 mm), which would allow for coupling and measurement of channels up to 300-μm deep without lenses.

The samples will be drawn into the analytical channels by capillarity. The capillary action and sample filling efficiency can be quantified by measuring the filling velocity using a less sensitive (barcode scanner) linear CCD. This CCD can easily track the bioluminescent fluid flowing through the channels and detect bubbles (dark areas). The velocity at which the solution travels through the channels will indicate the effectiveness of the sample loading. Channel shapes with the highest velocity will be chosen for testing sample loading into channels with lyophilized enzymes. Previous observations show that the capillary action within channels etched in silicon will work even at branching angles of 90° due to the thinness of the channels. The capillarity tests will also verify accurate sample volume loading because the fluid will flow to the end of the channel and will stop at the open end.

Analytical reagent solutions will be deposited into the ChemChip channels using inkjet printing and other low volume dispensing methods . The enzymes will be lyophilized and then enclosed in the channels by adhering glass over the channels as described above. Appropriate samples will then be added to the sample reservoir to initiate bioluminescent reactions with the lyophilized enzymes in the channels. Light from the channels will be measured as previously described. Preliminary reconstitution studies with dye and water in glass capillary tubes suggest that reconstitution can occur rapidly. Our preliminary experiments have shown that lyophilized bioluminescent enzymes reconstituted with ATP solutions produce enough light for CCD detection. Although lyophilized enzymes often have a hydrophobic contact angle, this is easily made hydrophilic by proper formulations with surfactants and hydrophilic polymers. (77) The channels and lyophilization techniques that produce the faster sample filling will decrease the sample filling speed as a factor in the diffusion and kinetic reactions within the channels. Channels and branches will be adjusted in the design if clogging or bubbles become an issue.

The reconstitution of the lyophilized enzymes with the analytical sample will be determined empirically by comparing the light output from the channel sides and ends as a function of lyophilized enzyme thickness, surfactants, and surface roughness (which may enhance mixing). The stability and kinetics of the lyophilized enzymes will greatly affect the efficiency of using bioluminescence as a method of analyte detection in a ChemChip. Light intensity verses time after lyophilization (ranging 1 week to 1 year, stored at different temperatures) will be compared to empirically test various vitamins and sugars and how they affect the stability if the bioluminescent enzymes.

Once the optimal channel shape is determined, bioluminescent intensity calibrations will be performed with ATP solutions ranging 0.1 to 10-μM in concentration using the same setup and methods described above. These tests will determine the lower limit of detection for ATP bioluminescent solutions. Similar calibration tests will be performed with a glucose detection bioluminescent liquid assay (glucokinase and firefly luciferase. During these tests, light scattering and interference will be analyzed by creating a ChemChip with two separate sample reservoirs. Each reservoir and their corresponding analytical channels will be filled with bioluminescent solutions of different ATP concentrations. Variability in light readings where two neighboring channels have two separate ATP solutions will indicate the amount of scatter and interference. Most of the interference, if any, is expected from any internal reflection within the glass cover slide. ChemChips with opaque covers (probably silicon), analytical channel spacing and digital signal processing will be tested to reduce or eliminate light scatter and interference. Coating the channels with thin metal films can also reduce scattering or interference

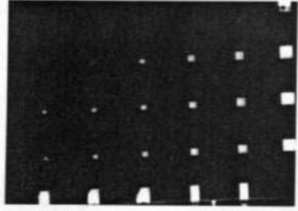

Figure 7: 20-sec integration of bioluminescence for the wafer that was etched 250-µm and coated with Cr. The micro reaction chambers seen here are the 750, 500, 400, 300, 250, and 200-µm wide squares.

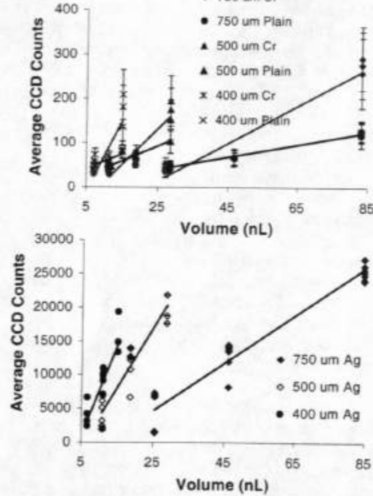

Figure 8: Average CCD counts from a 20-sec integrated CCD reading for different sample volumes. Data was plotted in sets for the same viewing area and separated according to Chromium coated and non-coated silicon (top graph). For each set of data, the increasing volume occurs from the increased etch depths. The intensity/volume slope is greater for Cr reflective substrates than for plain substrates. The bottom graph shows intensities from silver coated substrates that are about 100 times greater than that of the Cr coated substrates. Chambers sizes less than 400-µm wide are not shown, but the results were similar.

µRC Width (µm)	Intensity/Volume Slope Ratio: Chromium Verses Silicon	P Value	Intensity/Volume Slope Ratio: Silver Verses Chromium	P Value
750	3.02	0.00001	18.84	3.6E-18
500	2.37	0.002	8.31	3.7E-09
400	4.94	0.004	5.20	3.2E-05

Figure 9: The intensity efficiency is determined from the slopes, which represent amplification efficiency/volume for each size reaction chamber seen in Figure 8. The chromium enhanced the signal about 2-5 times that of the plain silicon substrate while silver enhanced the signal about 5-19 times that of chromium (16-54 times that of plain silicon).

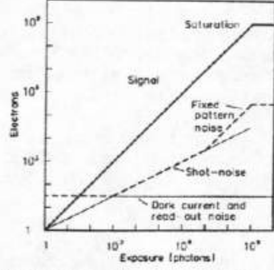

Figure 10: The relationship of photo-electrons produced in a CCD pixel from a given exposure to photons.

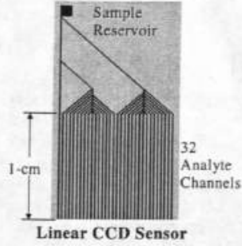

Figure 11: ChemChip with a linear array of long narrow analytical channels. The bioluminescence is integrated through the homogeneous fluid along the 1-cm channels. Channels of different depths, widths and lengths will be observed. A sample size of 1-µL can support 32 channels that are 1-cm long by 50-µm deep by 50-µm wide. The linear CCD sensors will measure the light output from the end of the channels.

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means of plotting and presenting data, including n-dimensional data, but which are not really widely applied in medicine or clinical chemistry.

We presented a preliminary example earlier. Fig. 6 showed a generic radar plot of the 7 key analytes in the diagnosis, management, and study of galactosemia. Note that by careful attention to the placement of the variables and the nature of the axes (linear, inverse, logrithmic, etc.), one can develop easily recognizable, identifiable patterns.

Fig. 12 is another example, this time for PKU. Here we used a data set provided by Reilly (58)of new born screening data, including not only Phe and Tyr but also Met and the branched chain amino acids (Val, Leu, Ileu). Normal values are represented by the small center circle (the value 1 represents the middle of the normal range). Those infants diagnosed with PKU had an average Phe 6 times that of normal, and a Tyr about 70% of normal. Hyperphenylalaninic patients have levels 2 to 3 times normal; they are generally not further studied or monitored. There are many such data sets in the literature; we propose to study them. Most have only been used for generic screening purposes and have not been used for more complete study.

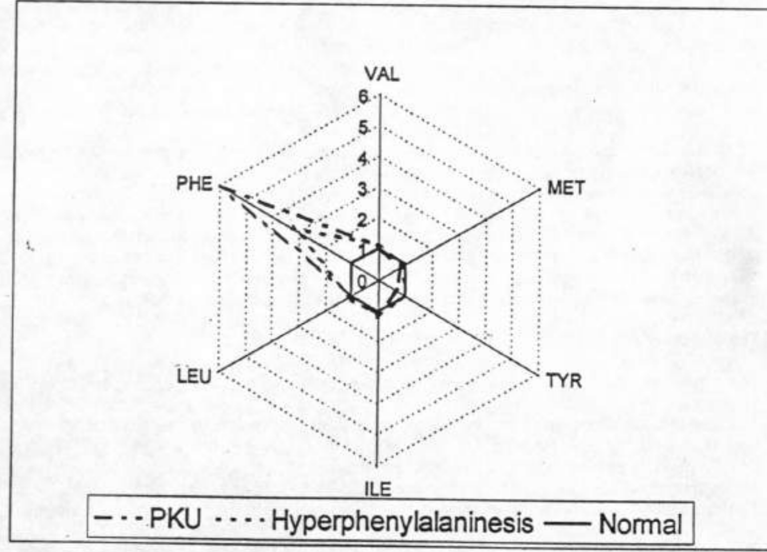

Figure 12 Radar plot of 3 of the Reilly, et al. (58) data sets; the average values of the PKU set, hyperPhe set, and the normal set are plotted so as to emphasize the differences. Optimal management of PKU is when the long abnormal Phe concentration "arrow" is reduced to the normal "circle" values.

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between channels. This may be necessary for the PDMS prototypes. At this point, variance and repeatability of measurements will be determined.

After calibration tests are performed with ATP and glucose solutions, the same calibration tests will be performed with blood and urine samples. Initial tests will be performed without red blood cell (RBC) filters. Bioluminescent glucose measurements with blood samples will be compared to a standard, calibrated glucometer as well as HPLC and spectrophotometer analysis. RBC filters commonly used in glucose test strips will be cut and placed in the sample reservoir as initial methods for measuring plasma constituents. Micropost filters will also be patterned into the channels (deep ionic etching into silicon wafers, or using deep ionic etched micropost filters as mold patterns in the PDMS ChemChips at the branches in order to perform analyte measurements in RBCs and plasma separately.

After the fabrication, microfluidics, and enzyme preparation techniques are developed and verified, other analytes will be added to the ChemChip. Initial ChemChip designs with 10 channels will gradually increase to 30 to 40 during the 5 year project. Analytes and the specific enzymes needed to measure those analytes by bioluminescence will be added and tested in the ChemChip prototype used for ATP and glucose. By using the same procedures, enzymes can be added to the ChemChip as custom panels are defined for PKU, then galactosemia, until a full panel of up to 40 various analytes are added to the ChemChip.

The graduate student primarily responsible for the design and development of the ChemChip, Daniel A. Bartholomeusz, has studied and applied micromachining under the direction of Dr. Bruno Frazier, now at Georgia Tech., and a consultant on this project.

Task 5: Data Analysis, Visualization, and Presentation

How can we deal with 40 different channels of chemical information? We will address what we might call the chemical "cockpit problem" . How can physicians, patients, and family members effectively deal with the interactions among tens and eventually hundreds of different metabolites, nutrients, and drugs? Fortunately, advances in data analysis, parameter presentation, and visualization -- coupled with appropriate modeling, simulation, and sensitivity analyses -- allow this challenge to be effectively addressed

A simple but highly useful approach to multi-dimensional "visualization" is the use of radar, spider, or star plots (all synonomous) utilizing radial, polar, or even spherical 3-D coordinate systems to present multi-dimensional data. We have used this in a preliminary way to simplify and to visualize the complexity of protein interfacial reactions.(5) We are now using it as a means to present multi-parameter clinical chemistry data so that the visual pattern generated by the locus of points on the spider plot is designed to reflect particular disease states and metabolic conditions. Although such plots are incorporated in some plotting and graphical analysis packages and software, and widely used in certain specific fields such as sensory assessment, they have not been widely applied in most other areas of science. There has been limited use in clinical medicine, demonstrating that such approaches have enormous potential.(7)

Perhaps the clearest example of an n- dimensional visualization method applied to clinical medicine and clinical biochemistry is the work of Cerra and co-workers dealing with the nutritional management of metabolic stress. Their study of the role of branched chain amino acids in the stress response (6, 31-33)presented the data in a unique radial or star plot, similar to those which we have used ibn our protein studies. There are many other very effective

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We will also experiment with the novel approaches of nDV, LLC, a local company whose focus is n-dimensional visualization. Their proprietary web-site is www.globalsvcs.com/ndv^ (please treat this as proprietary information). The web site has a full explanation and interactive examples.

nDV has developed several new intellectual structures which provide n-dimensional graphical viewing to aid in understanding of structure and relationships in arbitrarily many dimensions. The techniques are visually intuitive and mathematically tractable. nDV's version of Parallel Coordinate representation generates multi-dimensional display space images that look like overviews of mountainous terrain. As the system changes (for instance, in response to a new input), the terrain changes, and the evolution and propagation of this change is easily visualized at whatever scale the user desires.

n-DV's visualization tools build a digital model which can be viewed, animated, and manipulated to visually reveal relationships that might be in that data space. A display-space model is constructed from spreadsheet data, presenting a "Manhattan" view of the data; it shows what might be skyscrapers arranged around a rectangular grid of streets. Such 3-D Manhattan views are classically generated by spreadsheet graphing programs and mathematical programs such as Maple and Mathematica. The difference in n-DV's display space model is that the "avenues" are considered dimensions and the "streets" are considered rows or indexed value sets of the dimensions, and the "heights" of the skyscrapers are the values in each cell of the spreadsheet. This multidimensional perspective on what one is looking at is vital in the analysis and discernment of possible relationships and structures in the modeled data spaces.

n-DV's Display-space models also allow the superposition of several sets of data, one on top of the other, or aligned horizontally, to facilitate visual comparisons between sets of experiments or observations as represented by several separate spreadsheets worth of data..The display space models are built to be animated (viewed in motion). Motion of the model with respect to the viewer, or the motion of the viewer with respect to the model, is key to cognitive recognition of relationships that are not evident when looking at the static graphical Manhattan type models. n-DV also provides animation tools and capabilities uniquely useful in manipulating these models to facilitate discernment of relationships that may be within those models.

The objectives of these models include:

To be able to observe structure or relationships generated along, across, and among the coordinates of multidimensional.data;
To come to understand how these structures can be descriptive of relationships among or between the variables within data;
To help quantify those relationships whenever practical;
To learn what is important about the behavior of the "system" behind that data; and
To devise techniques for recommending further experiments or observations to be performed;

We will also work with Dr. Dwayne Westenskow, Professor of Bioengineering and Anesthesiology, principal investigator on a BRP project with Dr. Julio Bermudez from the School of Architecture. They are applying basic principles of 2D and 3D design (e.g., scale, shape, color, unity, etc.) and sound to produce "an interactive multi-sensory information system" that will allow us to more readily see and follow critical events in the operating room and critical care environment.

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Visualization is also a popular and productive activity in the radiology and medical imaging community. We will involve our local bioengineer/medical imagers and our other collaborators in discussions to quickly and efficiently learn what is available in their communities. The whole issue of perception of these visualization tools is critical—a subject also of interest to medical imaging.

We are interested in very simple ways to illustrate and help understand complex phenomena. We are interested in working with artists , cartoonists, etc. In the words of Martin Kemp and Richard Feynman(78): "Feynman diagrams look superficially like the simple graphics that physicists have uses for centuries. But they are devices of exceptional power. Within their space-time coordinates, Feynman was able to sidestep the long winded algebraic formulas that treated electrons and positrons separately. All the equations came together in one picture in a way that preceded and even directed calculation...
The diagrams mirror his conviction that "there is...a rythym and a pattern between the phenomena of nature which is not apparent to the eye, but only to the eye of analysis.... Such a potent grammar of diagrams and matching equations provides a marvelous tool."

And in Feynman's words:
"Strange! I don't understand how it is that we can write mathematical expressions and calculate what the thing is going to do without being able to picture it." (78)

A new NIH-NCRR Center at the Univ. of Utah (www.sci.utah.edu/ncrr/) will conduct research and development in advanced modeling, simulation, and visualization methods for solving bioelectric field problems. Chris Johnson, Rob MacLeod, and co-workers will create and disseminate a computational workbench to drive the development of a bioelectric problem solving environment. The programs will be robust enough to support expert-users and novices alike. With their system, researchers of bioelectric fields will be empowered to analyze their data, their methods, and the full range of their problem space in ways they had never before considered. The users will be free to apply their expertise where it makes the most sense—to steer an integrated computational system, to visually explore their data, to experiment with model parameters. When the tools integrate seamlessly, they fade into the background—the user is freed to concentrate on the problem at hand, rather than on the software tools. Such a seamless computational workbench is the ultimate goal. Although their tools are designed for bioelectricity studies, they clearly have a very wide applicability. We will work together to adapt and adopt their "computational workbench" to our needs.

Human Subjects: An IRB was approved on 21 December, 1999. The IRB number is 7786-99.

Vertebrate Animals – None

Literature Cited
List of Abbreviations –

ATP	adenosine triphosphate
BCCP	Biotin Carboxyl Carrier Protein
BPF	Bioprocessing Core Facility
BRP	Bioengineering Research
CCD	charge coupled device (in digital and video cameras)
CMOS	complementary metal oxide semiconductor
CRHCT	Cost Reducing Health Care Technologies

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CsA	cyclosporine A
ESRD	end stage renal disease
FDA	Food and Drug Administration
FK-506	tacrolimus
FMNH2	flavin mononucleotide (reduced)
G-1-P	galactose-1-phosphate
GFR	glomerular filtration rate
HPCC	High Performance Computing Center (Univ. of Utah)
HPLC	high performance liquid chromatography
IHC	Intermountain HealthCare
IPTG	isopropyl-1-thio-beta-D-galactopyranoside
IVD	in vitro diagnostics
LDH	lactate dehydrogenase
MRC	MicroReaction Chambers
NADH	nicotinamide adenine dincucleotide (reduced)
n-DV	n Dimensional Visualization
NSF	National Science Foundation
PCMC	Primary Children's Medical Center
PDH	phenylalanine dehydrogenase
PEP	phosphoenolpyruvate
PKU	phenylketonuria
PSI	Protein Solutions, Inc.
TDM	therapeutic drug monitoring

Web Site Addresses:

http://ir2lcb.cnrs-mrs.fr/~athel/mcafaq.htm
www.advimmuno.com
www.arup-lab.com
www.biodesign.com
www.diabetes.org
www.diabetes.org
www.edsci.com
www.ee.cua.edu/~winters/HCTWorkshop
www.expasy.ch/cgi-bin/search-biochem-index
www.galactosemia.org
www.healthtechcost.med.utah.edu
www.ihc.com
www.isotechnika.comwww.jdfcure.com
www.jdfcure.com
www.nidcr.nih.gov
www.nidcr.nih.gov
www.pkunews.org
www.polysciences.com
www.promega.com
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Consultants – three consultants: Frazier, Johnson, VanWagenen; their letters follow this page. Their biosketches are in the Biosketch Section.