APPENDIX B

NATIONAL SCIENCE FOUNDATION
PROPOSAL COVER SHEET
Phase I Document 1
STTR—Small Business Technology Transfer
Program Solicitation No. 93-162

TOPIC	ANALYTICAL CHEMICAL INSTRUMENTATION
PROPOSAL TITLE	Direct Reading, Quantitative Biosensors for ATP-Dependent Processes
NAME OF PROPOSING SMALL BUSINESS CONCERN	Protein Solutions, Inc.
ADDRESS (Including ZIP CODE)	390 Wakara Way, Room 31 Salt Lake City, Utah 84108
NAME OF RESEARCH INSTITUTION	University of Utah
ADDRESS (Including ZIP CODE)	2480 MEB Salt Lake City, Utah 84112
REQUESTED AMOUNT	$ 99,933
PROPOSAL DURATION	12 months
REQUESTED STARTING DATE	July 1, 1994

THE SMALL BUSINESS CONCERN CERTIFIES THAT:	YES	NO
1. It is a small business as defined in this solicitation.	X	
2. It qualifies as a socially and economically disadvantaged business as defined in this solicitation. FOR STATISTICAL PURPOSES ONLY		X
3. It qualifies as a women-owned business as defined in this solicitation. FOR STATISTICAL PURPOSES ONLY		X
4. It will exercise management direction and control of the performance of the STTR funding agreement.	X	
5. The agreement between it and the research institution concerning the allocation of rights is satisfactory to the small business concern.	X	
6. It will permit the government to disclose the title and technical abstract page, plus the name, address and telephone number of the corporate official if the proposal does not result in an award to parties that may be interested in contacting you for further information or possible investment.	X	
7. It will comply with the provisions of the Civil Rights Act of 1964 (P.L. 88-352) and the regulations pursuant thereto.	X	
8. It will perform _____ percent of the work and the collaborating research institution will perform _____ percent of the work as described in the proposal.	X	

PRINCIPAL INVESTIGATOR / PROJECT DIRECTOR	
NAME	R. Scheer
SOCIAL SECURITY NO.	253-35-1321
TELEPHONE NO.	(801) 585-3128

RESEARCH INSTITUTION INVESTIGATOR	
NAME	J.D. Andrade
SOCIAL SECURITY NO.	565-52-0772
TELEPHONE NO.	(801) 581-4379

OTHER INFORMATION		
PRESIDENT'S NAME	YEAR FIRM FOUNDED	NUMBER OF EMPLOYEES
J.D. Andrade	1988	AVERAGE PREVIOUS 12 MO.: 3 CURRENTLY: 4

PROPRIETARY NOTICE See Section 7.4 for instructions concerning proprietary information.
NOTE: The signed Certification Page must be submitted immediately following this Cover Sheet with the original copy only.

APPENDIX C

NATIONAL SCIENCE FOUNDATION
STTR—Small Business Technology Transfer Program

NSF 93-16

PROJECT SUMMARY

NSF AWARD NO.

NAME OF FIRM
Protein Solutions, Inc.

ADDRESS (Including ZIP CODE)
390 Wakara Way, Room 31
Salt Lake City, Utah 84108

PRINCIPAL INVESTIGATORS (NAME and TITLE)
R. Scheer, Research Scientist

TITLE OF PROJECT
Direct Reading, Quantitative Biosensors for ATP-Dependent Processes

TECHNICAL ABSTRACT (LIMIT TO 200 WORDS)

This project is an outgrowth of two different R & D efforts leading to two product lines originally targeted to science education: Bioluminescence and the Lableess Lab™. We now find that these two unique and very different technologies can be combined to produce what one might call analytical chemistry without instruments. The basic idea is to build the entire instrument into the sampling device.

Readout and detection utilizes light produced by bioluminescence. Specificity is provided by the use of enzymes. In this project we focus on direct analysis of ATP using firefly luciferase coupled with other enzyme-mediated biochemical processes which either produce or consume ATP.

Light intensity is a complex function of enzyme, substrate, and ATP concentrations and is a dynamic kinetic process. Light intensity is notoriously difficult to quantitate using the human eye. The sensor is therefore designed so as to produce light in a particular area of a two dimensional sample slide. It is the position of the light, rather than its intensity, which is related to substrate concentration. Man can directly detect the spatial position of a light source in a reproducible and quantitative manner, whereas his/her perception of the intensity of that light source is highly variable.

The sensors utilize stable luciferase/luciferin films coupled to a capillarity-based sample distribution system. The result will be specific, quantitative, rapid, direct reading, inexpensive and disposable analytical devices.

KEY WORDS TO IDENTIFY RESEARCH OR TECHNOLOGY (8 MAXIMUM)
Biosensor, Bioluminescence, Luciferase, ATP, Enzyme, Luminescence

POTENTIAL COMMERCIAL APPLICATIONS OF THE RESEARCH
Clinical measurements, home-care, education, biotechnology, personal diagnosis

their cell membranes (3-8). Trehalose apparently serves two major functions. In high concentration it tends to prevent phase separation and crystallization, and it tends to substitute for water normally hydrogen bonded to proteins and cell membranes thereby stabilizing their structures when the last molecules of water are removed by desiccation or drought. Trehalose is now being widely applied to the stabilization of enzymes and antibodies and is being studied for the stabilization of air dried cells, tissues, and organisms (3-6).

Mr. C.Y. Wang, a Ph.D. student in the Department of Bioengineering at the University of Utah, has been working on the trehalose stabilization of firefly luciferase for the past year under a contract between PSI and the Center for Biopolymers at Interfaces. Mr. Wang has succeeded in stabilizing firefly luciferase, ~~together with its substrate, luciferin,~~ in agarose gels for extended periods. More importantly, such agarose/luciferase/~~luciferin~~ gels can be completely dehydrated and desiccated, maintaining their clarity and transparency, and then rehydrated with full enzyme activity after extended periods. If such rehydrated gels are now exposed to ATP, they of course luminesce. We are already proceeding to develop this technology for educational and commercial purposes. It serves as the basis however, for the much more ambitious and significant project described in this application.

PSI has been working closely with the Center for Biopolymers at Interfaces and the Dept. of Bioengineering at the University of Utah for the past five years. In addition, the Dept. of Bioengineering has a wealth of experience in several technologies which are important to the proposed work. They have extensive experience in fluoroimmunosensors (37), thin film and waveguide optics, protein and enzyme immobilization (34, 36), protein and enzyme stabilization, protein and enzyme purification and characterization, and, more recently, the extensive experience in bioluminescence of both intact organisms and purified luciferases (35, 36), as well as some experience with dry reagent analytical systems. All of these are important and help provide the basic science foundation for this project. More recent studies dealing with capillarity and spreading, again primarily directed to dry reagent systems, as well as to protein separation for science education purposes, are also important components.

This very extensive multi- and inter-disciplinary background is what makes this project possible. PSI's existing Technology Transfer agreement with the University of Utah includes the technologies and topics ~~in this STTR~~ application ~~(Section O)~~.

Technical Approach/Rationale

The goal is to develop miniature analytical chemistry systems, specific for ~~particular aqueous~~ analytes which are quantitative, rapid, direct reading (by self luminescence), sensitive, with long shelf life, stable, disposable, and inexpensive.

~~In this project we are proposing to assess the feasibility of such sensors for ATP-dependent processes utilizing firefly luciferase. A future project will consider the application of bacterial luciferase to sense NADPH-dependent processes.~~ Because there are literally hundreds, perhaps thousands, of enzymes which are involved with ATP consumption or ATP production, most of which are specific to another chemical substrate, for example, glycerol, glucose, etc., these "front end" enzymes permit the development of individual sensors or sensor channels for each of those substrates (28, 29, 13-17, 23, 24).

In this ~~Phase~~ I feasibility application we do *not* consider the front end enzymes. Rather, we will focus on the enabling technology to measure ATP concentration.

variety of sensors already exist for measuring ATP through the use of firefly luciferase (13, 15). However, these require sophisticated light detection and calibration systems, because they rely on light intensity.

Our unique and novel approach is to produce a spatial distribution of the emitted light which is proportional to the analyte concentration. The human photon detector is therefore used primarily to assess *spatial position* rather than absolute light intensity. This ~~will be~~ achieved by carefully controlling the luciferase concentration in the sensing gel and by modulating, tuning, and "filtering" the ATP concentration through the use of a second ATP consuming enzyme. This ATP "consumase" is selected to have a very rapid ATP turn over, and to serve as an ATP concentration regulator to the sensor. The ATP consumase is deposited in different spatial regions of the one or two dimensional sensor at different concentrations. At the top of Figure 1 we show a layer of ATP consumase immobilized in an agarose gel and containing its specific substrate, glycerol in the case of glycerol kinase (29). ~~The optimum ATP consumase to be used is one of the objectives of this study~~.

A sample containing ATP is distributed and diffuses into the ATP consumase gel layer, at which time the ATP is partially consumed and a lower level of ATP then enters the luciferase gel layer, which of course contains the optimum concentration of the luciferin substrate. As the ATP interacts with the luciferase, photons are emitted which are then detected by the operator viewing from the top of the page. The middle panel shows that the device consists of five channels (A, B, C, D, E), each with a different consumase concentration. For a uniform input of ATP into the consumase gel channels, each channel delivers a different ATP concentration to the uniform luciferase layer. This results in the light emission pattern shown on the bottom. Depending on the specific consumase concentrations, the luciferase concentration, and the concentration of ATP, the light output will be a maximum where the consumase concentration is minimum and will be a minimum where the consumase concentration is maximum.

By appropriate design of these concentration ratios, and by appropriate selection of a consumase with the appropriate turnover rate, which is matched to the delivery of ATP into the consumase gel, a specific spatial illumination pattern is obtained. The human eye is very good at detecting changes in contrast and spatial position.

Figure 2 shows three ATP concentrations, five consumase channels, and the result of three different luciferase concentrations. ATP concentration is schematically represented by the length of the arrows and is shown being delivered to the consumase gel layer, containing five channels of different consumase concentration. That results in a change in the ATP concentration delivered to the luciferase gel. That ATP concentration is shown being delivered to a uniform luciferase gels of three *different* luciferase concentrations. The figure thus shows three different sensors, each exposed to three different ATP concentrations. There is obviously an increase in the light intensity as a function of increased ATP and for luciferase concentrations; more importantly is the fact that the spatial position, the inflection between high and low light output, changes as a function of ATP concentration. This means that a *spatial position is directly correlated with ATP level.* Spatial position can be measured by an operator viewing the device without special instruments and without great concern for dark adaptation or specific intensity values. There are of course significant kinetic issues which must be addressed -- such analyses are major parts of ~~the~~ Phase II ~~objectives and research plan.~~

and bacterial luciferase enzymes, respectively. Such biosensors generally involve fiberoptic or other waveguided means of delivering the luminescence to a device which can accurately measure light intensities (13,15-17, 23-28, 31). Although one of the most portable and most sensitive photon detectors available to the analytical chemist is his or her own eye, it is notoriously difficult to calibrate for accurate measurements of even relative light intensity. The human two dimensional photon detection system, however, can reliably and accurately measure changes in spatial position.

We propose to utilize this characteristic as the readout system for a quantitative, inexpensive, disposable, analytical device for the analysis of ATP and a range of other bio-chemicals involved in ATP-dependent enzyme processes.

E. BACKGROUND

Protein Solutions, Inc. (PSI) was founded in early 1988 to develop and produce innovative science educational products and personal sensors. We now manufacture ~~Night Life: Science in the Dark™, a~~ bioluminescence-based science kit designed for ~~upper elementary~~ students ~~and~~ their teachers. ~~We also manufacture and distribute Galaxsea™, a bioluminescent product for the high school and adult market which introduces them to the wonders and beauty of bioluminescence (35).~~

Bioluminescence is an enzyme dependent chemical oxidation process which results in photon emission (1, 2). The photoproteins involved in these processes, the luciferase series of oxidative enzymes, are now readily available in inexpensive form, produced by recombinant means (10).

During the last 5 years, PSI has been deeply involved in all aspects of bioluminescence, including several studies dealing with its application to biosensors. We have funded a study at the Center for Biopolymers at Interfaces at the University of Utah ~~(the Research Institution partner in this STTR)~~, on the behavior of firefly luciferase, the photo protein/enzyme responsible for catalyzing bioluminescence in the North American firefly. We have studied its adsorption at solid/liquid interfaces, air/water interfaces, and its denaturability or stability in solution (36). We have recently initiated a similar set of studies dealing with bacterial luciferase, the enzyme responsible for bioluminescence in various species of marine bacteria. These studies, together with our commercial work on bioluminescent phytoplankton (the dinoflagellate *Pyrocystis lunula*) have provided a deep and diverse background in practical bioluminescence.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (12, 18, 25). Since the firefly luciferase reaction is dependent on an adenosine triphosphate (ATP) co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Likewise, bacterial luciferase utilizes a ubiquitous chemical in bioenergetic pathways, and has therefore been widely applied for biosensing applications. Up until very recently such applications were frustrated by the relative instability of these enzymes and the difficulty in incorporating them in practical and reliable biosensors (31, 19, 13).

~~A year ago Protein Solutions, Inc.~~ became interested in a new science education product based on another unique biological phenomena -- the ability of certain plants and animals to survive almost total desiccation for extended periods and to spring back to life when rehydrated, a phenomenon called anhydrobiosis (8). In developing our new science educational product, Resurrection™, we of course learned that ~~most~~ of these plants and animals depend on a unique disaccharide, trehalose, for their ability to withstand severe desiccation stress without denaturation of their proteins and enzymes or disruption of

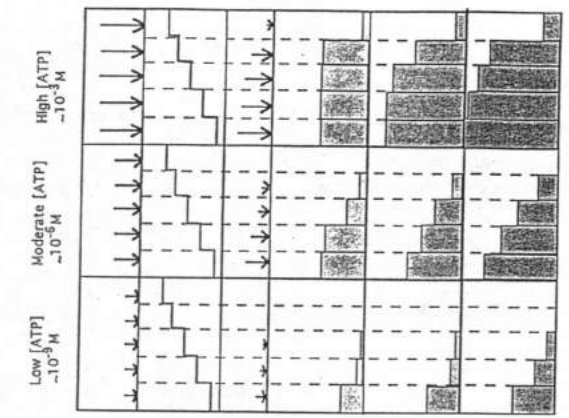

Fig.2 Spatial detection of analyte concentration. The ATP consumase serves as a spatial "filter" for ATP. By tuning analyte concentration to the turnover rate of the analyte consuming enzymes, a spatial pattern is produced which is related to applied analyte concentration. [Luciferin]=1 mM, turnover rate:ATPase/luciferase=100

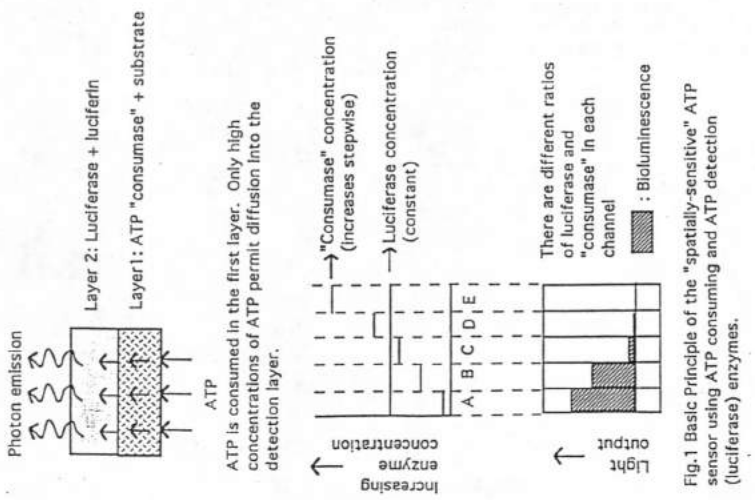

Fig.1 Basic Principle of the "spatially-sensitive" ATP sensor using ATP consuming and ATP detection (luciferase) enzymes.

There are many possible design concepts for such a sensor. Figure 3 shows a dual sensor (two luciferase concentrations in a rectangular configuration); other designs will be considered during the Phase I study. We rely on dry reagent technologies and a capillarity-driven analyte sample delivery system (22, 38). On the top of Figure 3 the sample is deposited over a rectangular area designed to accommodate a constant volume; the sample is distributed by wicking uniformly along the long dimension of the sample. In the right half of the rectangular device we assume the luciferase gel has a high concentration with a low concentration on the left half.

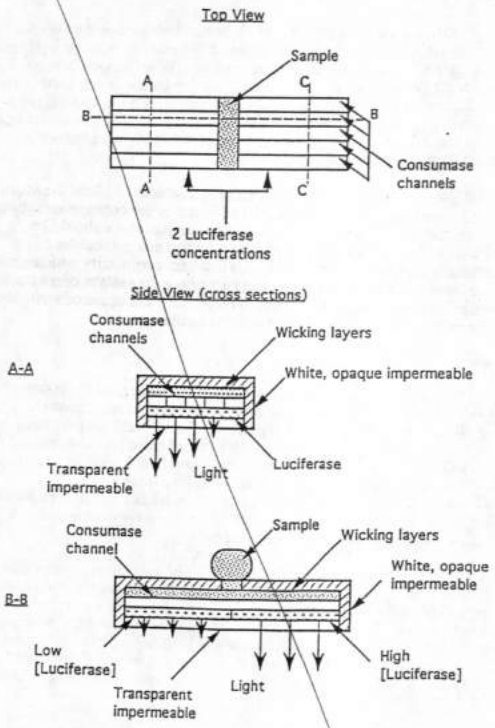

Fig.3 Preliminary design concept for the spatially-sensitive ATP sensor involving dry reagent technologies and a capillary-based analyte (sample) delivery mechanism.

different, unique bioluminescent systems in nature have yet to be extensively applied to analytical chemistry (1), with the possible exception of calcium monitoring based on aequorin and related photoproteins. It has been estimated that there are at least 30 and may indeed be several hundred different, unique, bioluminescent systems involving different luciferases, different luciferins and different co-factors (1, 2, 20). That all remains to be explored and applied.

F. PHASE I RESEARCH OBJECTIVES

The Phase I research objectives are summarized in Table 1 which is divided up into three general categories: Fundamental Data (Obj.1-4); Device Design and Prototype (Obj. 5-7); Testing and Evaluation (Obj. 8-9); and Preliminary Work for Phase II (Obj. 10-11). Responsibilities are indicated in the two far right columns, the basic work via the University of Utah subcontract, and the more applied and device oriented work at Protein Solutions, Inc. The eleven tasks are briefly explained here and described in more detail in the next section.

Table 1. Summary of Phase I Research Objectives.

Task	(Applied) PSI		(Basic) U of U	
P.I.	(Scheer)		(Andrade)	
Fundamental Data:				
1. Protein Preparation/Characterization	—		(Wang)	X
2. Luciferase gels/"consumase"/trehalose/ agarose/starch	(postdoc)		(Min)	X
3. Gel spreading/preparation/stability	(Scheer)	X	--	
4. Capillarity -- sample delivery and transport	(postdoc)	X	(Min)	X
Device Design and Proto-type:				
5. Simulation of capillarity, gel diffusion and enzyme reaction rates	(Scheer postdoc)	X	(Dryden, Wang)	X
6. Gel/Enzyme Patterning -- Printing	(postdoc)	X	—	
7. Device Design and Fabrication	(Scheer)	X	—	
Testing and Evaluation:				
8. Optical Imaging	(Scheer)	X	(Wang)	X
9. Device Testing	(Scheer)	X	—	
Preliminary Work for Phase II:				
10. "Front End" Enzymes -- Recommendation	(postdoc)	X	(Andrade)	X
11. Bacterial Systems -- Recommendation	—		(Min)	X

1. *Protein Preparation and Characterization.* This task will be done by students at the University of Utah, using the facilities at the Center for Biopolymers at Interfaces. They are experienced with the production of firefly luciferase by recombinant means from E. coli (10). Preliminary studies will also be performed on the culture of marine, bioluminescent bacteria, and the extraction and purification of the bacterial luciferase. In addition the ATP "consumases" will also be purified and characterized. At the present time this is likely to be glycerol kinase (29), although a number of

In the other dimension of the device we show five consumase channels. As one goes from the center of the device to the right or left in the top view, we have to understand that the ATP is being delivered by wicking and that the amount delivered will be a function of distance from the center. The side views are AA and BB sections. AA is a section through the five consumase gels in the left hand portion of the device. Here you see the wicking layer which has distributed the sample to this part of the device. The ATP will then move into the various consumase channels where it will be acted upon by the consumase, resulting in different concentrations of ATP being delivered to the uniform luciferase gel. The different intensities of photon emission are indicated by the length of the photon arrows.

The BB cross section is a longitudinal cross section; we have not attempted to show the kinetics of wicking. Depending on the feasibility studies to be done in Phase I, we may require a timing step prior to luminescence observation. It may also be possible to design the device so the timing step is not critical. Going back to the top view, looking from the bottom, imagine a pattern of light emission, varying with time, to the right and to the left, and varying in spatial position along the AA and CC axes. The intensities and spatial positions along AA and CC will be different because these two sections of the device involve different luciferase concentrations.

A number of other variables in the analysis and optimization include the actual sample size to be delivered, the rate of wicking, the diffusion of the appropriate substrates into the two gels, and the specific time or times when the luminescence should be observed by the user. Although one could argue that there are many variables and therefore considerable complexity in the system, it is that very complexity which allows us to design and develop a device in which one can simply *see* the analyte concentration by virtue of an optical luminescent pattern, visually detected by the operator without the need for other instruments. That is the potential and the challenge.

Anticipated Benefits:

Patterns and images are much easier to detect, to remember, and to process than numbers. Man is exquisitely constructed to deal with visual patterns and pattern recognition. The design, development, and application of analytical devices which are self luminescing and generate a specific pattern which can be directly correlated or related to analyte concentration, has enormous potential benefits. In addition to greatly simplifying routine measurements in a range of analytical, environmental, biotechnological, and medical/diagnostic laboratories, such technology has the potential for being applied widely in schools, education, and in the home for a variety of monitoring, diagnostic, and other measurement purposes.

Looking at health care alone, a one trillion dollar per year industry in the United States 2, 12, 32), chemical/clinical diagnostics accounting for perhaps 5-10% of that, and with the growing role of analytical chemistry in the biotechnological and environmental areas, it has been estimated that the biosensor industry is in the range of one to ten *billion* plus dollars per year (32). With the growing interest in home-based clinical chemistry measurement and a growing movement towards home care and personal care, it is clear that the opportunity for simple, inexpensive, disposable, and specific analytical chemistry devices should grow dramatically (32).

It is important to note that our spatially sensitive ATP sensing system is also adaptable to the bacterial luciferase NADPH system (26-28, 31, 12, 17-19) and to a variety of chemiluminescent systems (12, 21). It is also important to note that, with the exception of the firefly and bioluminescent marine bacteria, most of the 30 or so other

other possibilities will be examined. This part of the project will prepare the proteins in suitable quantities for the other components.

2. *Proteins Immobilized in Agarose ~~and/or Starch Gels~~ Containing Trehalose.* Trehalose is a stabilizer for both the gel and the protein, permitting it to be totally dehydrated and stored, and then rehydrated at a later date to full enzyme activity. Optimum conditions for the preparation of gels with the various enzyme concentrations desired will be explored. This is technology developed at the University of Utah and will be transferred to PSI during the Phase I work.

3. *Gel Spreading, Preparation, and Stability.* The gel solution (sol) has to be applied and spread on suitable supports in a uniform and homogeneous manner. At present this is done at the University by a dipping or casting process, that technology will now be performed at PSI. PSI will also develop technology of preparing thin gel layers by various printing processes (see Task 6 below).

4. *Capillarity: Sample, Delivery and Transport.* The sample containing the analyte will be deposited on a suitable device and spread across the appropriate portions of the gel by a capillarity and rehydration process. This technology is already applied in existing dry reagent chemistry diagnostic kits (38). There is experience at the University in this area, largely on thin layer chromatographic processes involving aqueous media for protein separation. That work will be initially done at the University and then transferred to PSI.

5. *Simulation of Capillarity Gel Diffusion and Enzyme Reaction Rates.* The selection of optimal conditions will be greatly aided by a theoretical model and simulation which capillarity-based analyte delivery, the diffusion of analyte through the gel, and its interaction with enzymes. The various turnover numbers will be simulated. Existing enzyme kinetic models, diffusion models and capillarity models will be used. ~~This work will also be done initially at the University by Mr. Paul Dryden who has extensive experience in mathematical modeling and computer simulation, and by Dr. Rob Scheer, PI of the project, who also has considerable modeling and simulation experience.~~

6. *Gel Enzyme Patterning and Printing.* J. Andrade at the University experimented with ink jet printing for the deposition of proteins for biosensor applications some five years ago. Screen printing is now quite common in the biosensor community. We are confident that we can "ink jet" print and spray print protein/agarose and/or protein/starch solutions under conditions which will form the patterns desired for the multi-channel sensor.

7. *Device Design.* Based on the results of Tasks ~~1-6~~ above, and continued extensive discussion, the design for a prototype device will be finalized within the first four months of the project. The first decision is whether to use a rectangular device with spreading and analyte delivery in two directions, or to use a circular, ~~radial multi-directional~~ device. ~~The decision will rest in large part on the results of Task five.~~

8. *The Prototype Devices Will Be Tested and Evaluated Through Bioluminescence Intensity, Spatial Distribution, and Ability to detect and determine ATP Concentration.* This will be done at the University using the optical imaging laboratory at the Center for Biopolymers at Interfaces, as well as visually with a number of disinterested volunteers. CCD camera patterns and visually determined patterns will be compared to see if any particular training or experience is required for disinterested, unaided observers.

9. *Device Testing.* Task 8 will be extended to a range of ATP concentrations of normal analytical significance and interest. In addition, short term storage, dehydration, stability, and reproducibility experiments will be performed. This will be done in the third quarter in preparation for a thorough reanalysis of Tasks 1-9, development of an optimized device design, and development plan for the Phase II proposal.

10. *Preliminary Work Will Assess the Advisability of Incorporating Other Enzymes for the "Consumase" Portion of the Sensor, as Well as a Range of ATP-Dependent Enzymes to Extend the Analysis to Other Substrates.* The so-called "front end" enzymes which would be incorporated in a *third gel layer* on the front end of the sensor would thus make the sensor sensitive for other enzyme substrates. This will be an important component of the Phase II application.

11. *A Preliminary Analysis of the Bacterial Luciferase System and a Comparison of its Advantages and Disadvantages with the Firefly Luciferase System.* At this point there is no intention of doing extensive studies with the bacterial system or making it an important part of the Phase II project, but nevertheless, given its importance and its complementarity to firefly luciferase, it is important that such analysis be performed. This task, as well as Task 10, will be done primarily on the University sub-contract.

Basically, Tasks 1-4, the fundamental work which is the underpenning of the project, will be completed largely in the first quarter. Tasks 5-7 in the second quarter, Tasks 8-9 in the third quarter, and Tasks 9-11 in the fourth quarter. The work is distributed roughly 1/3 at the University of Utah, and 2/3 at PSI. The close working relationship between PSI and the University of Utah, and the close physical proximity of the two entities guarantees a strong and successful collaboration and technology transfer process.

G. PHASE I RESEARCH PLAN

1) Protein Preparation and Characterization.

Although much of our work with firefly luciferase has utilized firefly-derived material, we have initiated studies on the cultivation of E-coli using an appropriate plasmid which expresses firefly luciferase. This recombinant firefly luciferase is then extracted from the E-coli and subjected to a series of purification steps. The protocol for firefly luciferase expression in E-coli and for purification are readily available and will not be repeated here (10).

The ATP "consumase" which we use initially is glycerol kinase derived from *E. coli*. This enzyme transfers a phosphate group from ATP to glycerol to produce glycerol 3 phosphate and ADP. Its V-Max, the number of ATP molecules processed per minute per milligram of pure protein, is over 100 times that of firefly luciferase (29). Our preliminary analysis suggests that a very major difference in V-Max is required between the two enzymes to obtain the spatial sensitivity and localization of luminescence which is critical to this unique sensor concept.

Commercial and reference preparations of glycerol kinase are available for comparison and standardization purposes. We will examine a number of other kinases involving the intraconversion of ATP, ADP, and AMP. There are many such enzymes available, many of which are commercially available in inexpensive form.

applied them in thin film form, studied liquid transport and distribution in such films, and utilized them for protein separation. In fact, Protein Solutions, Inc. has another project to develop a simple, 2-dimensional *Protein Card* for the separation of tear proteins, urine peptides, and possibly saliva proteins for educational applications.

The University and the PSI groups both have substantial experience and a strong commitment to capillarity-based sample delivery and separation processes. J. Andrade, P.I. of the University sub-contract, is a surface chemist with 25 years of teaching and research experience and will personally supervise the capillarity-based experimental and simulation studies.

5) Simulation of Capillarity, Diffusion, and Enzyme Reactions.

The modeling and simulation studies will be broken into three parts during the first half of Phase I and then combined into a comprehensive, more complete model in the second half of Phase I. The parts include:

Capillarity. The parameters required for these simulations are either available in the literature or can be readily determined experimentally using various dyes coupled with automatic time-dependent imaging of the fluid profile. Indeed this has already been done for a number of other studies.

Diffusion of ATP in the partially hydrated and fully hydrated gels will be estimated based on data in the literature for molecular diffusion through such gels (30). Because of the wide application of these agaroses for protein and enzyme encapsulation/immobilization, there is a substantial database which already exists and which we can adapt for our analyses.

Enzyme reaction rates will be determined experimentally for our particular preparations and will be used in standard enzyme kinetic models employing single enzyme/dual substrate and dual enzyme/single substrate competitive analysis.

The problem of course becomes more complex when we attempt to couple and combine the three individual phenomena to a complete simulation of our proposed device. Nevertheless, we are confident that the modeling exercise will prove to be very helpful in enhancing our understanding of the various phenomena and their coupling, and in the design of experiments with which to determine the optimum characteristics and specifications of our prototype devices.

6) Gel Patterning and Printing.

Some years ago we utilized ink jet printing for multi-channel biosensor applications -- proteins could be deposited in various patterns on paper substrates. This technology was later modified to produce protein patterns on rigid planar substrates. The process is to spray the luciferase/luciferin/trehalose/agarose solution, followed by a drying step. The process is repeated, under spatial control, for the agarose/consumase layer.

Given our earlier experience with protein patterning using moderately viscous protein solutions, we anticipate no serious problem with this part of the project. We expect that we will have to develop a local heating system for the cartridge and tubing on the ink jet to prevent the agarose solution from gelling prior to contact with the support which is being "printed." Technical staff responsible for this work have already had

The proteins will be characterized by high pressure liquid chromatography and by non-denaturing and denaturing polyacrylamide gel electrophoresis. The initial protein purification and characterization work will be accomplished through the University of Utah sub-contract.

2) Enzyme/Trehalose Gel.

Our experience on the stabilization of firefly luciferase in gels has been limited to a specific kind of agarose. There is a report from a Russian group (11) that starch is effective for bacterial luciferase; we have some very preliminary work suggesting that other gels may work under certain conditions. The goal is to softly immobilize the enzyme in the gel, generally in the presence of its luciferin substrate, and allow ATP to diffuse into the gel from an external source. This is the most effective method for biosensing applications.

We expect that trehalose is the most effective additive for stabilizing the protein in polysaccharide gels, i.e., agaroses, celluloses, dextrans, etc. Although we have extensive experience with the stabilization of firefly luciferase in trehalose/agarose gels, we have little experience with the consumase or kinase enzymes at this point. Trehalose has, however, been used by others for the stabilization of a variety of enzymes, including kinases. (6) Therefore, we anticipate no problem with the preparation of the kinase/trehalose/agarose gel. The gels will be prepared with different enzyme/trehalose ratios. The enzyme activity can be directly measured by delivering reference amounts of ATP and then detecting the resulting ATP diffusion into a luciferase gel, essentially the basis of our biosensor concept.

We will also experiment with dual enzyme gels, that is, trehalose/agarose gels containing both luciferase and kinase. Although this was not mentioned in the earlier schematic drawings, it is an approach which may indeed have some advantages.

Gels would be dried at different rates, subjected to storage under different temperature conditions, rehydrated at different rates, and evaluated for enzyme activity. We will also do a number of studies in which the rehydration is coupled with ATP analyte delivery, thereby evaluating the kinetics of hydration, enzyme activity recovery, and luminescence signal generation.

3) Gel Spreading.

We'll improve the process of preparing thin gel films by a controlled dipping process followed by rapid cooling to induce gelation. We will also experiment with a conventional horizontal casting process. Surface treated Mylar films are commercially available, designed for optimum adhesion to thin agarose coatings, and are widely used for thin layer electrophoresis applications. We have considerable experience with these films already. They appear to be in almost ideal support for our enzyme films and perhaps for the prototype devices.

4) Capillarity: Sample Delivery and Transport.

Most existing dry film tabs used for clinical diagnostic applications use a wicking/capillarity means of delivering the analyte to the sample area and volume. We have performed studies on the spreading of analyte containing solution, on glass fiber filters, and on protein sample delivery via capillarity on cellulose supports used for thin layer chromatography (39). We have also studied films prepared from ion exchange particles and size exclusion particles, used in special areas of chromatography, and

extensive experience using ink jet printing and related patterning techniques for chemical and biosensor development.

7) Device Design and Fabrication.

The understanding and experience derived from Tasks 1-6 in the first 6 months of the project will permit us to proceed to optimize our device design and fabricate prototypes. We anticipate no serious problems here.

We will also perform a number of assessments of user preference. That is we will simulate the area and spatial distribution of luminescence and the expected photon flux of luminescence intensity for a variety of observers, aimed at assessing the optimum pattern in spatial distribution to permit the observer to derive the most information regarding ATP concentration from light spatial position. Based on these perception studies and the data from Tasks 1-6, a set of prototype devices will be constructed.

8) Optical Imaging.

The prototype devices will then be exposed to appropriate concentrations of ATP and luminescence imaged with a sensitive CCD camera, capable of near single photon detection, as a function of time. We will thus be able to follow the development of the capillarity, diffusion, and enzyme reaction in real time on prototype devices, and on the various components of those prototype devices. The sensitive, time dependent, two dimensional optical imaging will allow us to really characterize these prototypes, and to quantitatively determine the photon yield as a function of spatial position and time. This work will be performed at the University of Utah in the Optical Imaging Laboratory.

Our preliminary calculations and suggestions suggest that the photon flux will be such that it can be detected of course by a normal dark adapted eye. During the Phase II program we expect to further enhance and optimize the device, and to design a packaging and inexpensive viewing port, which would enable the observation to be made with the non adapted eye. The results from Task 8 will be used to further enhance and improve the device, and for the construction of the second generation of prototypes for further testing.

9) Device Testing.

The second generation prototypes will then be subjected to extensive testing using a range of ATP concentrations, analyte sample volumes, reading times, and degree of dark adaptation of the viewer. We will then be in a position to objectively assess practical feasibility and, if feasibility is determined, as expected, to proceed with the more definitive and extensive Phase II research and development activities.

10) Front End Enzymes.

A preliminary assessment of ATP independent enzymes for the front end of the generic sensor will be made in preparation for the Phase II effort. Again, there is an extensive literature and experience with such coupled enzyme systems for specific biosensor applications (13, 28). We will simply apply that knowledge and experience to our very unique sensor prototypes to develop enhanced and optimum prototypes for an array of different analytes during Phase II.

(normally 0 – 25 gr/liter)

carbohydrate

9. *Device Testing.* Task 8 will be extended to a range of ~~ATP~~ concentrations of normal analytical significance and interest. In addition, short term storage, dehydration, stability, and reproducibility experiments will be performed. ~~This will be done in the third quarter in preparation for a thorough reanalysis of Tasks 1-9, development of an optimized device design, and development plan for the Phase II proposal.~~

10. *Preliminary Work Will Assess the Advisability of Incorporating Other Enzymes for the "Consumase" Portion of the Sensor, as Well as a Range of ATP-Dependent Enzymes to Extend the Analysis to Other Substrates.* The so-called "front end" enzymes which would be incorporated in a *third gel layer* on the front end of the sensor would thus make the sensor sensitive for other enzyme substrates. This will be an important component of the Phase II application.

11. *A Preliminary Analysis of the Bacterial Luciferase System and a Comparison of its Advantages and Disadvantages with the Firefly Luciferase System.* At this point there is no intention of doing extensive studies with the bacterial system or making it an important part of the Phase II project, but nevertheless, given its importance and its complementarity to firefly luciferase, it is important that such analysis be performed. This task, as well as Task 10, will be done primarily on the University sub-contract.

Basically, Tasks 1-4, the fundamental work which is the underpenning of the project, will be completed largely in the first quarter. Tasks 5-7 in the second quarter, Tasks 8-9 in the third quarter, and Tasks 9-11 in the fourth quarter. The work is distributed roughly 1/3 at the University of Utah, and 2/3 at PSI. The close working relationship between PSI and the University of Utah, and the close physical proximity of the two entities guarantees a strong and successful collaboration and technology transfer process.

G. PHASE I RESEARCH PLAN

1) Protein Preparation and Characterization.

Although much of our work with firefly luciferase has utilized firefly-derived material, we have initiated studies on the cultivation of E-coli using an appropriate plasmid which expresses firefly luciferase. This recombinant firefly luciferase is then extracted from the E-coli and subjected to a series of purification steps. The protocol for firefly luciferase expression in E-coli and for purification are readily available and will not be repeated here (10).

The ATP "consumase" which we use initially is glycerol kinase derived from *E. coli*. This enzyme transfers a phosphate group from ATP to glycerol to produce glycerol 3 phosphate and ADP. Its V-Max, the number of ATP molecules processed per minute per milligram of pure protein, is over 100 times that of firefly luciferase (29). Our preliminary analysis suggests that a very major difference in V-Max is required between the two enzymes to obtain the spatial sensitivity and localization of luminescence which is critical to this unique sensor concept.

Commercial and reference preparations of glycerol kinase are available for comparison and standardization purposes. We will examine a number of other kinases involving the intraconversion of ATP, ADP, and AMP. There are many such enzymes available, many of which are commercially available in inexpensive form.

applied them in thin film form, studied liquid transport and distribution in such films, and utilized them for protein separation. In fact, Protein Solutions, Inc. has another project to develop a simple, 2-dimensional *Protein Card* for the separation of tear proteins, urine peptides, and possibly saliva proteins for educational applications.

The University and the PSI groups both have substantial experience and a strong commitment to capillarity-based sample delivery and separation processes. J. Andrade, P.I. of the University sub-contract, is a surface chemist with 25 years of teaching and research experience and will personally supervise the capillarity-based experimental and simulation studies.

5) Simulation of Capillarity, Diffusion, and Enzyme Reactions.

The modeling and simulation studies will be broken into three parts during the first half of Phase I and then combined into a comprehensive, more complete model in the second half of Phase I. The parts include:

Capillarity. The parameters required for these simulations are either available in the literature or can be readily determined experimentally using various dyes coupled with automatic time-dependent imaging of the fluid profile. Indeed this has already been done for a number of other studies.

Diffusion of ATP in the partially hydrated and fully hydrated gels will be estimated based on data in the literature for molecular diffusion through such gels (30). Because of the wide application of these agaroses for protein and enzyme encapsulation/immobilization, there is a substantial database which already exists and which we can adapt for our analyses.

Enzyme reaction rates will be determined experimentally for our particular preparations and will be used in standard enzyme kinetic models employing single enzyme/dual substrate and dual enzyme/single substrate competitive analysis.

The problem of course becomes more complex when we attempt to couple and combine the three individual phenomena to a complete simulation of our proposed device. Nevertheless, we are confident that the modeling exercise will prove to be very helpful in enhancing our understanding of the various phenomena and their coupling, and in the design of experiments with which to determine the optimum characteristics and specifications of our prototype devices.

6) Gel Patterning and Printing.

Some years ago we utilized ink jet printing for multi-channel biosensor applications -- proteins could be deposited in various patterns on paper substrates. This technology was later modified to produce protein patterns on rigid planar substrates. The process is to spray the luciferase/luciferin/trehalose/agarose solution, followed by a drying step. The process is repeated, under spatial control, for the agarose/consumase layer.

Given our earlier experience with protein patterning using moderately viscous protein solutions, we anticipate no serious problem with this part of the project. We expect that we will have to develop a local heating system for the cartridge and tubing on the ink jet to prevent the agarose solution from gelling prior to contact with the support which is being "printed." Technical staff responsible for this work have already had

11) The Bacterial System.

The bacterial luciferase system has been used in conventional biosensors for a wide range of NADPH and FMN-dependent enzymes. We feel there is considerable information and experience here, which will aid us in the further research and development of our firefly luciferase dependent system. We fully expect that, as we develop experience with bacterial luciferase in our other programs, we will present a future research and development proposal on a complementary array of bio-sensors using bacterial luciferase.

References:

1. Special issue, "Bioluminescence in the Sea," *Naval Research Reviews* **45** (1993) (#2).
2. J. Lee, "Bioluminescence: Biochemistry for Fun and Profit," in D.P. Valenzeno, ed., *Photobiological Techniques*, Plenum, 1991, pp. 297-321.
3. T. Arakawa, S.J. Prestelski, W.C. Kenney, and J.F. Carpenter, "Factors Affecting Short-Term and Long-Term Stabilities of Proteins," *Adv. Drug Delivery Reviews* (1993), in Press.
4. J.F. Carpenter and J.H. Crowe, "Modes of Stabilization of a Protein by Organic Solutes During Desiccation," *Cryobiology* **25** (1988) 459.
5. J.H. Crowe, L.M. Crowe, D. Chapman, "Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose," *Science* **223** (1984) 701.
6. C. Colaco, et al., "Extraordinary Stability of Enzymes Dried in Trehalose," *BioTech.* **10** (1992) 1007.
7. B.P. Gaber, et al., "Interaction of Trehalose..." Molecular Modeling," in A.C. Leopold, ed., *Membranes, Metabolism, and Dry Organisms*, Cornell Univ. Press, pp. 231.
8. J.H. Crowe, et al., "Anhydrobiosis," *Ann. Rev. Physiol.* **54** (1992) 579.
9. A.F. Dukhovich, N.N. Ugarova, and I.V. Berezin, "Firefly Luciferase as a Complex of Protein with Lipids," *Dokl. Acad. Nauk SSSR* **289** (1986) 231-233.
10. K. Wood, Ph.D. Dissertation, Department of Chemistry, University of California, San Diego, 1989.
11. V. Kratysuk, Abstract, *Conference on Bioluminescence*, Maui, Dec., 1993.
12. L.J. Kricka, et al., *Anal. Applic. of Bioluminescence and Chemiluminescence*, Acad. Press., 1989.
13. A. Roda, et al., "Coupled Reactions for the Determination of Analytes and Enzymes Based on ... Luminescence," *J. Biolum. Chemilum.* **4** (1989) 423-435.
14. F.H. Harold, *The Vital Force: Bioenergetics*, W.H. Freeman, Co., 1986.
15. L.J. Blum, et al., "Design of Luminescence Photobiosensors," *J. Biolum. Chemilum.* **4** (1989) 543-550.
16. P.R. Coulet, "Luciferase-based Sensors," in Proc. Second World Congress on Biosensors, *Biosensors 92*, Elsevier Adv. Tech., pp. 2-9.
17. P.J. Worsfold and A. Nabi, "Bioluminescent Assays...," *Anal. Chim.Acta* **179** (1986) 307.
18. T.O. Baldwin, et al., "...Immunoassay...Using Bacterial Luciferase," *Meth.Enzym.* **133** (1986) 248-264.
19. N.N. Ugarova and O.V. Lebedeva, "Immobilized Bacterial Luciferase..." *Appl Biochem Biotech* **15** (1987) 35-51.
20. J.W. Hastings, "Bioluminescence in Bacteria and Dinoflagellates," in *Light Emission by Plants and Bacteria*, Acad. Press, 1986, pp. 363-398.
21. I. Bronstein and A. Sparks, "...Enzyme Immunoassays With Chemilum...," in R.M. Nakamura, et al., eds., *Immunochemical Assays and Biosensor Technology*, Amer. Soc. Microbiol., 1992. pp. 229-250.
22. R.F. Zuk, et al., "Enzyme Immunochromatography," *Clin. Chem.* **31** (1985) 1144-1150.

H. COMMERCIAL POTENTIAL

Please refer to the later part of Section E relating to anticipated benefits. J. Andrade and the group at the University of Utah Center for Biopolymers at Interfaces, together with the University's Office of Technology Transfer, have had considerable experience with major diagnostic and sensor companies. We expect considerable interest in this technology. PSI expects to enter into an appropriate agreement for commercial funding of the development as Phase III. PSI will itself fund and market these sensors for educational and home use.

I. RELATED RESEARCH

This was covered in detail in Sections D and E.

J. PERSONNEL

Dr. Robert Scheer, Principle Investigator, received his Ph.D. in Materials Science and Engineering in 1993 and has been working for PSI. He is Principle Investigator of PSI's Labless Lab™ in Polymer Materials, an SBIR Phase I from the National Science Foundation (33). Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. Although he has not had an extensive biochemistry or biomaterials background, he is certainly learning fast. His abbreviated vita is appended to this section.

Dr. Joseph Andrade is President, founder, and Chief Scientific Officer of PSI. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, particularly with their behavior at surfaces and interfaces (32, 37). About 5 years ago he became quite interested in bioluminescence and particularly in the firefly and bacteria luminescence systems. He will provide much of the interfacial biochemistry, bioluminescence, and biosensor expertise required. Joe is three quarter time Professor of Materials Science and Bioengineering at the University of Utah and is P.I. of the University of Utah sub-contract. His abbreviated vita is also attached.

Mr. C.Y. Wang is a graduate student working under Joe Andrade's supervision at the University of Utah. Mr. Wang has worked on the firefly luciferase system for nearly four years and will be completing his Ph.D. studies on this system in late 1994 or early 1995. He will thus be available during a major part of the Phase I award to conduct some of the firefly luciferase-based studies and to transfer his rich, comprehensive expertise on that system to the others involved in the project. There is a distinct possibility that Mr. Wang would elect to stay on in a post-doctoral capacity on the Phase II project.

Mr. Dong Min is also a graduate student under Joe Andrade's direction, working on bacterial luciferases. He is following in Mr. Wang's footsteps and basically doing a similar series of studies, but with the bacterial luciferase rather than the firefly system. He will work closely with Mr. Wang to learn the techniques and methodologies, which he can apply to the bacterial problem, as well as being responsible for the luciferase studies when Mr. Wang completes his studies.

Both students will be partly supported on the University sub-contract. The University of Utah and Protein Solutions, Inc. have a Technology Transfer agreement and a full potential Conflict of Interests/Disclosure Policy. PSI also has a research contract with the University of Utah through which other specialized studies which require University equipment and access can be conducted.

23. U. Wollenberger, et al., "Biosensor for ADP...," *Anal. Lett.* **20** (1987) 657-668.

24. U. Wollenberger, et al., "Enhancing Biosensor Performance Using Multienzyme Systems," *TIBTech* **11** (1993) 255-262.
25. D. Griffiths and G. Hall, "Biosensors -- What Real Progress is Being Made?," *TIBTech* **11** (1993) 122-130.
26. A. Nabi and P.J. Worsfold, "...Assays...Using Immobilized Bacterial Luciferase," *Analytical Letters*, **22** (1989) 1861-1871.
27. S.M. Gautier, et al., "Bioluminescence-Based Fibre-Optic Sensor With Entrapped Co-Reactant...," *Analytica Chimica Acta* **243** (1991) 149-156.
28. A.Nabi and P.J. Worsfold, "Determination of Ethanol and Alcohol Dehydrogenase Using Co-immobilised Bacterial Luciferase and Oxidoreductase," *Analyst* **111** (1986) 531-533.
29. J.W. Thorner and H. Paulus, "Catalytic and Allosteric Properties of Glycerol Kinase from *E. coli*," *J. Biological Chem.* **248** (1973) 3922-3932.
30. For exameple, B.H. Schneider, et al., "Microminiature Enzyme Sensors for Glucose and Lactate Based on Oxygen Electrodes," *Sensors and Actuators*, **B1** (1991) 565-570.
31. N.N. Ugarova, et al., "Bioluminescent Microassay of Various Metabolites Using Bacterial Luciferase Co-immobilized with Multienzyme Systems," *Analytical Biochemistry* **173** (1988) 221-227.
32. J.D. Andrade, ed., *Medical and Biological Engineering in the Future of Health Care*, U. of Utah Press, 1994, see chapters by I. Karube and J. Andrade.
33. Andrade, J.D. Andr R.J. Scheer, "Applying 'Intelligent Materials for Materials Education: The Lableess Lab™," *Proc., 2nd Annual Conference on Intelligent Materials*, Tech. Publ. Co., 1994, in press.
34. J.D. Andrade, et al., "Proteins at Interfaces: Principles, Problems, and Potential," chapter in *Interfacial Behavior of Bioproducts*, J. Brash and P. Wojciechowski, eds., Dekkar, 1994, in press.
35. J.D. Andrade, et al., "Using Bioluminescence for Integrated Science Education," in A.A. Szalay, et al., eds., *Bioluminescence and Chemiluminescence: Status Report*, Wiley, 1993, pp. 69-73.
36. C.Y. Wang and J.D. Andrade, Ibid., pp. 99-103.
37. J.D. Andrade, et al., "Immuno-Biosensors: The Clinical Chemistry and Coagulation Laboratory on a Chip," in Y. Sezai, ed., *Artificial Heart: Biomation in the 21st Century*, Saunders, 1992, pp. 89.
38. The Kotak Ekta-Chem clinical chemistry tabs are a good example.
39. J. Luo, M.S. Thesis, U. of Utah, in preparation.

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EDUCATION

Ph.D. in Materials Science and Engineering, September 1993, University of Utah, Salt Lake City, UT. Dissertation emphasis: Mechanical, interfacial, and surface study of composite materials.

B.S. in Mechanical Engineering, 1989, Duke University, Durham, NC. GPA 3.76. Emphasis: Fracture mechanics and failure analysis of polymeric materials.

UNIVERSITY HONORS

National Science Foundation Fellow,
University of Utah Graduate Research Fellow,
Dean's List Duke University, Academic All American
Duke University Magna Cum Laude,
Scholastic Societies: Tau Beta Pi and Pi Tau Sigma.

EXPERIENCE

Principle Investigator
Protein Solutions, Inc. Salt Lake City, UT. 1994 - present. Directed research for the design and implementation of novel science education materials.

Research Assistant
University of Utah, Salt Lake City, UT. 1989 - 1994. Tested mechanical properties of polymers and composites, studied surfaces and interfaces, tested adhesive bonds on the microscopic scale, and developed stress analyses related to materials testing.

Instructor/Tutor
University of Utah and Salt Lake Community College, Salt Lake City, UT. 1991 - present. Planned, instructed, and graded for undergraduate physical science classes. Served as tutor and teaching assistant.

Engineering Technician
Sandia National Laboratory, Albuquerque, NM. Summer, 1988 and Summer, 1989. Designed engineering experiments for failure analysis of ceramic materials, and extensively researched current experimental techniques for determining material fracture toughness. Designed engineering experiments for strength testing of brittle materials, and performed CAD. Interacted with diverse engineering disciplines on a major research project.

AFFILIATIONS: American Society for Mechanical Engineers, ASM International, The Minerals, Metals, and Materials Society, American Physical Society, The Center for Biopolymers at Interfaces

PUBLICATIONS:

Scheer, R.J. and J.A. Nairn. "Variational Mechanics Analysis of Stresses and Failure Analysis in Microdrop Debond Specimens." *Composites Engineering*, Vol. 2, No. 8, pp. 641-654, 1992.

Scheer, R.J. Ph.D. Dissertation, "An Energy Based Analysis of Fiber-Matrix Adhesion." University of Utah, 1993.

Andrade, J.D. and R.J. Scheer. "Applying 'Intelligent' Materials for Materials Education: The Lableess Lab™." *Proc., 2nd Annual Conference on Intelligent Materials*, Tech. Publ. Co., 1994, in press.

FILE COPY

1. **Title:** NATIONAL SCIENCE FOUNDATION STTR PHASE I FINAL REPORT

2. **Program Official/Organization:** Michael Crowley (Darryl Gorman)/DMII

3. **Program Name:** STTR/DMII

4. **Award Dates:** **Effective Date:** 08/15/94
   Expiration Date: 07/31/95

5. **PI/PD Name:** Robert J. Scheer

6. **Company, Address, Phone Number, and FAX Number:**

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Phone: (801)596-2675
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Salt Lake City, UT 84121
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FAX: (801)583-9301

7. **Award Number:** DMI-9413561

8. **Project Title:** Direct Reading, Quantitative Biosensors for ATP-Dependent Processes.

9. **Phase II Intentions:** We intend to submit a Phase II proposal.

This material is based upon work supported by the National Science Foundation under award number DMI-9413561. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the National Science Foundation.

Proprietary Information is contained on page 22 of this report.

A. PHASE I RESEARCH OBJECTIVES (VERBATIM)

The Phase I research objectives are divided up into three general categories: Fundamental Data (Obj. 1-4); Device Design and Prototype (Obj. 5-7); Testing and Evaluation (Obj. 8-9); and Preliminary Work for Phase II (Obj. 10-11). The basic work was performed via the University of Utah subcontract, and the more applied and device oriented work was performed at Protein Solutions, Inc. The eleven tasks are briefly explained here:

1. *Protein Preparation and Characterization.* This task will be done by students at the University of Utah, using the facilities at the Center for Biopolymers at Interfaces. They are experienced with the production of firefly luciferase by recombinant means from E. coli (1). Preliminary studies will also be performed on the culture of marine, bioluminescent bacteria, and the extraction and purification of the bacterial luciferase. In addition the ATP "consumases" will also be purified and characterized. At the present time this is likely to be glycerol kinase (2), although a number of other possibilities will be examined. This part of the project will prepare the proteins in suitable quantities for the other components.

2. *Proteins Immobilized in Agarose and/or Starch Gels Containing Trehalose.* Trehalose is a stabilizer for both the gel and the protein, permitting it to be totally dehydrated and stored, and then rehydrated at a later date to full enzyme activity. Optimum conditions for the preparation of gels with the various enzyme concentrations desired will be explored. This is technology developed at the University of Utah and will be transferred to PSI during the Phase I work.

3. *Gel Spreading, Preparation, and Stability.* The gel solution (sol) has to be applied and spread on suitable supports in a uniform and homogeneous manner. At present this is done at the University by a dipping or casting process, that technology will now be performed at PSI. PSI will also develop technology of preparing thin gel layers by various printing processes (see Task 6 below).

4. *Capillarity: Sample, Delivery and Transport.* The sample containing the analyte will be deposited on a suitable device and spread across the appropriate portions of the gel by a capillarity and rehydration process. This technology is already applied in existing dry reagent chemistry diagnostic kits (3). There is experience at the University in this area, largely on thin layer chromatographic processes involving aqueous media for protein separation. That work will be initially done at the University and then transferred to PSI.

5. *Simulation of Capillarity Gel Diffusion and Enzyme Reaction Rates.* The selection of optimal conditions will be greatly aided by a theoretical model and simulation which includes capillarity-based analyte delivery, the diffusion of analyte through the gel, and its interaction with enzymes. The various turnover numbers will be simulated. Existing enzyme kinetic models, diffusion models and capillarity models will be used. This work will also be done initially at the University by Mr. Paul Dryden who has extensive experience in mathematical modeling and computer simulation, and by Dr. Rob Scheer, PI of the project, who also has considerable modeling and simulation experience.

6. *Gel Enzyme Patterning and Printing.* J. Andrade at the University experimented with ink jet printing for the deposition of proteins for biosensor applications some five years ago. Screen printing is now quite common in the biosensor community. We are confident that we can "ink jet" print and spray print protein/agarose and/or protein/starch solutions under conditions which will form the patterns desired for the multi-channel sensor.

7. *Device Design.* Based on the results of Tasks 1-6 above, and continued extensive discussion, the design for a prototype device will be finalized within the first four months of the project. The first decision is whether to use a rectangular device with spreading and analyte delivery in

10. Abstract :

Project Summary:

a) This project was undertaken in response to the need for a direct reading biosensor, capable of real time measurement of bacterial contamination, enzyme activity or substrate activity. Readout and detection utilize **light produced by bioluminescence**. Specificity is provided by the use of enzymes. In this project we focus on direct analysis of adenosine triphosphate (ATP) using firefly luciferase coupled with other enzyme-mediated biochemical processes which either produce or consume ATP.

Light intensity is a complex function of enzyme, substrate, and ATP concentrations and is a dynamic kinetic process. Light intensity is notoriously difficult to quantify using the human eye. A person can directly detect the spatial position of a light source in a reproducible and quantitative manner, whereas his/her perception of the intensity of that light source is highly variable. The sensor is therefore designed to produce light in a particular area of a two dimensional sample slide. The sensor correlates the *position* of the light, rather than its *intensity*, with substrate concentration.

The sensors utilize stable luciferase films which are coupled to a capillarity-based sample distribution system. A specific, quantitative, rapid, direct reading, inexpensive and disposable analytical devices has been shown feasible in this phase I project.

Commercial Applications:

b) There are several major market areas with enormous commercial potential. Sensitive, direct reading, ATP-based, intrinsically luminescent, dipstick-type sensors will have major applications in clinical chemistry, personal diagnostics (home health care), environmental monitoring (water quality), microbiological monitoring (for sterile conditions such as food/dairy processing), biotechnology process control, education, and other fields.

two directions, or to use a circular, radial multi-directional device. The decision will rest in large part on the results of Task five.

8. *The Prototype Devices Will Be Tested and Evaluated Through Bioluminescence Intensity, Spatial Distribution, and Ability to detect and determine ATP Concentration.* This will be done at the University using the optical imaging laboratory at the Center for Biopolymers at Interfaces, as well as visually with a number of disinterested volunteers. CCD camera patterns and visually determined patterns will be compared to see if any particular training or experience is required for disinterested, unaided observers.

9. *Device Testing.* Task 8 will be extended to a range of ATP concentrations of normal analytical significance and interest. In addition, short term storage, dehydration, stability, and reproducibility experiments will be performed. This will be done in the third quarter in preparation for a thorough reanalysis of Tasks 1-9, development of an optimized device design, and development plan for the Phase II proposal.

10. *Preliminary Work Will Assess the Advisability of Incorporating Other Enzymes for the "Consumase" Portion of the Sensor, as Well as a Range of ATP-Dependent Enzymes to Extend the Analysis to Other Substrates.* The so-called "front end" enzymes which would be incorporated in a *third gel layer* on the front end of the sensor would thus make the sensor sensitive for other enzyme substrates. This will be an important component of the Phase II application.

11. *A Preliminary Analysis of the Bacterial Luciferase System and a Comparison of its Advantages and Disadvantages with the Firefly Luciferase System.* At this point there is no intention of doing extensive studies with the bacterial system or making it an important part of the Phase II project, but nevertheless, given its importance and its complementarity to firefly luciferase, it is important that such analysis be performed. This task, as well as Task 10, will be done primarily on the University sub-contract.

B. RESEARCH SUMMARY

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (4-6). Since the firefly luciferase/luciferin reaction is dependent on an adenosine triphosphate (ATP) co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Using luminometers and photomultiplier tubes, sensitivities to 10^{-11}mol/l are commercially available (7).

Under a contract between Protein Solutions, Inc. and the University of Utah's Center for Biopolymers at Interfaces (CBI), Mr. C.Y. Wang has been working on the trehalose stabilization of firefly luciferase for the past two years. Mr. Wang and coworkers have succeeded in stabilizing firefly luciferase in agarose gels for extended periods (8). These gels maintain their optical clarity in the dehydrated state, and can be rehydrated after extended periods with high levels of activity. With the awarding of this grant, Mr. Wang, and Dr.'s Scheer and Andrade have extended this gel preservation technique to apyrase and have developed a device for measurement of ATP concentration based on the spatial position of light output. Apyrase (an enzyme important in current ATP detection technology) is utilized to consume unwanted ATP (9).

Our unique approach to ATP detection is to produce a spatial distribution of the emitted light proportional to the analyte (ATP) concentration. The human photon detector can then be used to assess spatial position rather than absolute light intensity. This positioning is achieved by carefully controlling the ATP concentration using an ATP "filter", a second ATP consuming enzyme, apyrase. Apyrase has a turnover rate approximately 100 times faster than luciferase and serves as an ATP concentration regulator to the sensor.

Measurements of enzyme and substrate activity after prolonged gel storage were performed for different gel preparation and storage conditions. These measurements gave us the information necessary to decide which enzymes or substrates could be successfully stored and which preservation method was preferred.

After learning that both luciferase and apyrase could be stored for prolonged periods in a dehydrated state, refinement of the luciferase and apyrase concentration levels for appropriate readout were performed. Appropriate concentration levels were determined through a systematic examination of light output relative to the concentrations of luciferase, apyrase, and ATP. Each of the gel components including the luciferase and apyrase enzymes, and the analyte components including the luciferin substrate and coenzyme A were reviewed. We were looking for the highest light output for a given ATP concentration. A concentration of luciferase in the range of 0.1mg/ml, luciferin in the range of 0.5mM, coenzyme A in the range of 0.5mM, and apyrase concentration dependent on the range of ATP to be measured were determined

Finally wicking, capillarity, and optical studies to determine proper device geometry for fast delivery of analyte to the sensor gel spots were performed. These studies gave us the information necessary to decide on a likely test strip geometry. We were looking for a geometry which gave us a bright readout as well as fast analyte delivery.

These experiments have resulted in the following prototype design. The luciferase/apyrase enzymes are deposited as circular spots along one dimension of the device, each dot of increasing apyrase concentration. The physical support for the device is a strip of filter paper sandwiched between two polyester films with an agarose coating. One end of one of the films has an opening to allow the delivery of the analyte. (See figure 1.)

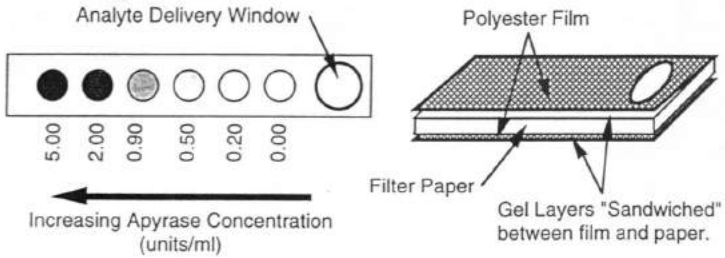

Figure 1. Diagram of increasing apyrase concentration in consecutive sensor dots and schematic of device design.

The analyte (containing the ATP sample to be measured, luciferin, and coenzyme A) is delivered to the analyte delivery window of the direct reading sensor and readout takes place in three minutes. This design takes advantage of both the wicking properties of the filter paper and the capillary action of the gel film sandwich. The test strip geometry allows sample delivery to each gel "dot" within one second of analyte delivery. The time limiting factor of the reaction is the rehydration of the enzyme gel itself.

The concept is based on the fact that, for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a "measurable" light output. Above that concentration, light is visible; below that ATP concentration, no light is measured. If a uniform concentration of ATP is delivered to a series of sensors (dots), they will either produce a measurable signal or an immeasurable signal.

By filtering or consuming the ATP before it reacts with the luciferase we control the intensity of the light at each position along the sensor. A high concentration of ATP will still produce light even at the higher apyrase concentrations because enough ATP remains after

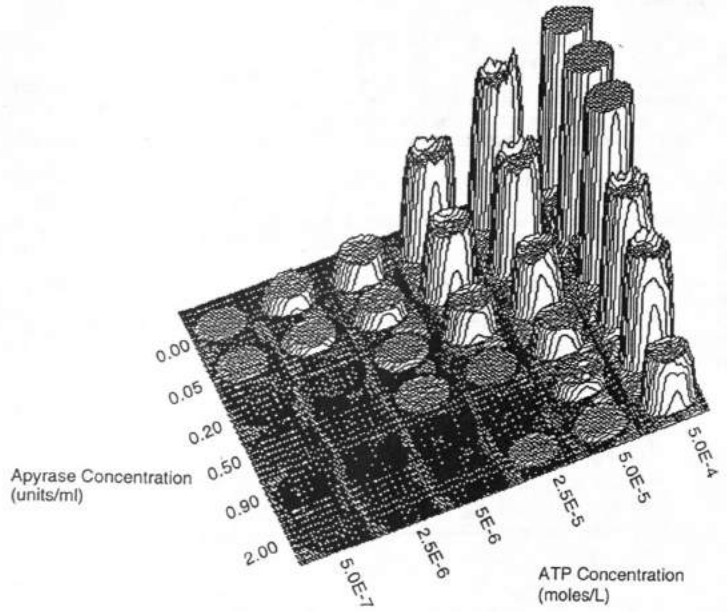

Figure 3. Typical CCD 3-D profile of light intensity for a six by six detector array.

We have begun preliminary studies into the use of hexokinase as an enzyme "specific" to glucose. In its conversion of glucose to glucose-6-phosphate, hexokinase consumes ATP.

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

Results of this study show that front end enzymes can be used to measure substrate concentration.

The measurement of ATP concentration itself is of limited commercial interest. However, the possibility of using this technology as an indicator of the presence of bacteria or other microorganisms, based on the release of intracellular ATP, is widely recognized (4,7,9-12). Hundreds, perhaps even thousands, of enzymes exist which are involved with ATP consumption or ATP production, most of which are specific to another chemical substrate, for example, glycerol, glucose, etc. These "front end" enzymes permit the development of individual sensors or sensor channels for each of those substrates (2,10-17). These substrates, in turn, permit the development of individual sensors for each of the enzymes.

Commercially, this technology has application in the food and dairy industry for rapid measurement of total microbial contamination. Other uses include household use for detection of bacteria and other microbes on eating surfaces, in the bathroom and in the water. A more complete analysis of commercial potential is in the Commercialization Plan at the end of this report.

consumption to produce a measurable light output. A low concentration of ATP will produce light only at the lowest apyrase concentrations because at higher apyrase concentrations all of the ATP is consumed before it reacts with the luciferase. (See figure 2.)

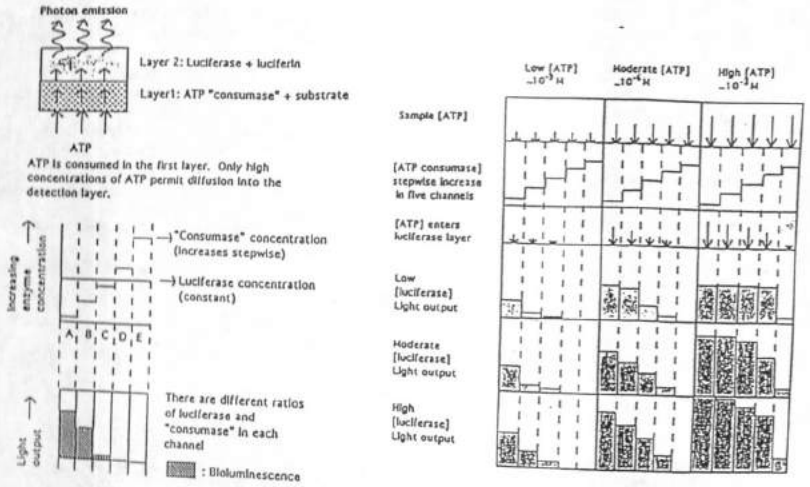

Figure 2. Conceptual drawing of separate device channels showing effect of different apyrase concentrations and different ATP concentrations

To measure an ATP concentration, a sample containing ATP is distributed to the luciferase/apyrase gel at which time the ATP is consumed by the two competing enzymes. Because the turnover rate of apyrase is two orders of magnitude higher than that of luciferase, the apyrase quickly moderates the ATP concentration. The amount of ATP which reacts with the luciferase to produce light depends on both the initial ATP concentration and the concentration of apyrase.

To demonstrate and test the dependence of light output on both of these concentrations, we produced 2-D (two dimensional) gradients of ATP concentration ($5x10^{-4}$ to $5x10^{-7}$ mol/l ATP) versus apyrase concentration (2 units/ml to 0 units/ml). (See figure 3.) This is a positive surface profile directly related to light intensity. The image was captured with a CCD camera. With high initial concentrations of ATP, a large concentration of apyrase is required to modulate the light intensity. With low initial concentrations of ATP, only small concentrations of apyrase are required to modulate the light intensity. The goal of such modulation is to generate a specific light cutoff point indicating a specific concentration of ATP in the analyte solution.

C. RESEARCH DETAILS

1. Protein Preparation/Characterization. *Objective: To characterize the enzyme activity of luciferase for use in the spatial detection device.*

Both native (purchased from Sigma) and recombinant firefly luciferase were characterized at the University of Utah's CBI under the direction of Dr. Joe Andrade. Mr. C.Y. Wang performed the actual protein preparation and characterization (18-22).

Recombinant protein preparation was carried out in CBI labs at the University of Utah.

(proprietary information included on final page)

Characterization involved gel electrophoresis to determine molecular weight and purity of the proteins, and photomultiplier tube (PMT) measurement of light intensity as a measure of activity.

Molecular weight of the proteins is estimated by the method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Figure 4 shows the developed gel from the electrophoresis study. Both the Sigma and the recombinant (BCCP) luciferase showed a high degree of purity, with only minimal difference in molecular weight owing to the BCCP tag on the recombinant protein.

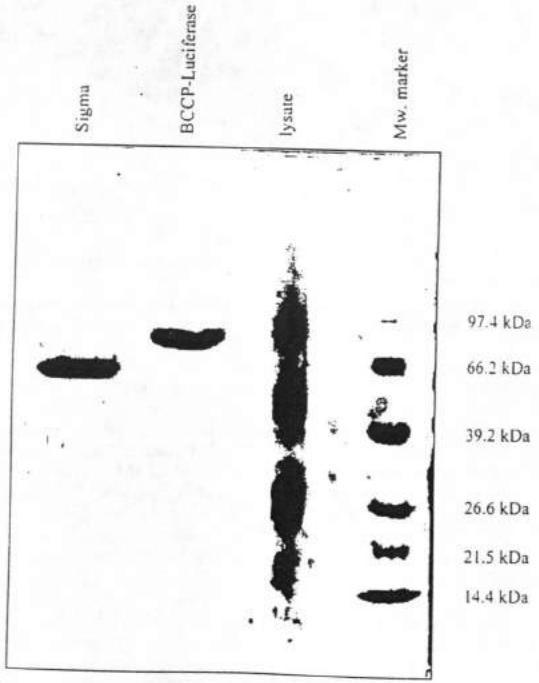

Figure 4. Electrophoresis Gel.

Figure 5 shows the relative activities of the recombinant and native luciferases (the avidin was part of another study by Mr. Wang). Three findings are important from this study. The luciferase activity drops dramatically above 40°C; this is the result of irreversible protein denaturation. Second, the highest luciferase activity is found in the range of 25°C. Finally, there are no consistent or major differences between the activities of the native and recombinant luciferases.

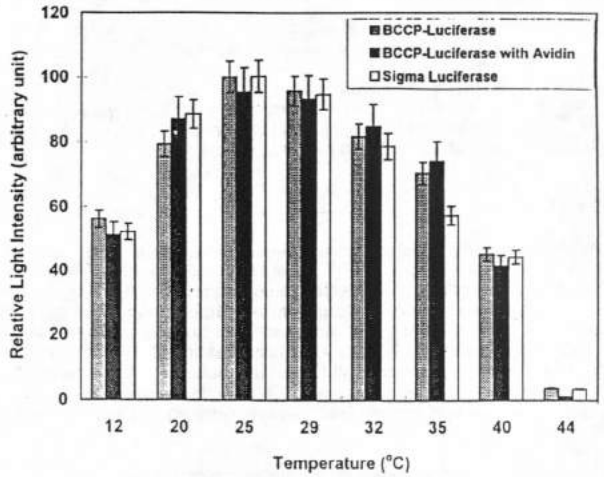

Figure 5. Enzyme intensity comparison between native and recombinant luciferase.

Because of its widespread use for such purposes, apyrase (from potato) was the ATP "consumase" chosen for initial study (7,9). After preliminary characterization and stability studies, we determined that apyrase met the kinetic requirements specified for the ATP concentration moderator (i.e. that it have an enzyme reaction rate several orders faster than luciferase).

Results: Both native and recombinant luciferase exhibit the highest levels of activity at temperatures between 25 and 30°C. No significant difference in activity was detected between native and recombinant luciferase. Because the turnover rate of apyrase is 100 times faster than that of luciferase it will serve as an excellent ATP "filter".

2. Luciferase Gels–Preservation/Storage. *Objective: To determine the optimum gel preparation and storage conditions for maintenance of enzyme activity.*

Hydrated agarose gels containing different combinations of luciferase/apyrase/luciferin/coenzyme A were prepared, dehydrated, stored and rehydrated under several conditions to measure enzyme/substrate stability and to determine optimum preparation and storage conditions for the gel.

Two luciferases were studied. Luciferase from *Photinus pyralis* was obtained from Sigma Chemical Company and recombinant luciferase purified in CBI labs were used in the preservation and gel entrapment study. The Sigma luciferase solution was prepared with 0.45M GlycylGlycine buffer, pH 7.8. The recombinant luciferase solution was prepared with glycerol.

D-luciferin, free acid was purchased from Molecular Probes, Inc. This substrate, along with ATP, is required for the luminescence reaction.

3. Gel Spreading/Preparation/Stability. *Objective: To determine a suitable physical support for the dehydrated gel and optimize sol/gel application methods.*

By including trehalose in the gel at a concentration of 100mg/ml the agarose gel maintained optical clarity and both the luciferase and apyrase enzymes were stabilized upon drying.

We examined the wetting characteristics of the sol on several supports: opaque polystyrene microtitration plates, transparent and colorless agarose coated polyester film (GelBond® film, 0.1mm thick, FMC Corporation, made by 3M), glass capillary tubes, cotton threads, and Whatman filter paper number 1.

The polystyrene plates were opaque, white microtitration plates manufactured by Dynatech Laboratories, Inc. The plates were charged with 50µl of the enzyme sol (0.1 mg/ml luciferase in a 1% agarose containing 100 mg/ml trehalose) using a manual pipet. The sols evenly covered the bottom of the wells. Final test preparations included cooling to gelation temperature, and dehydration at 20°C and 20% relative humidity for 24 hours. The dried gels were transparent and the enzyme maintained high activity as evidenced by CCD imaging of luminescence.

The transparent polyester film was purchased with a precoated layer of agarose on one side. The film was charged with 50µl of enzyme gel using a manual pipet. The sols spread evenly on the film test strips, forming circular dots approximately 8mm in diameter. Final test preparations of the gel on the polyester film were the same as those described above. The dried gels were transparent and the enzyme maintained high activity as evidenced by CCD and PMT imaging of luminescence.

The capillaries were 75 mm long, with an I.D. of 1.1 mm and a thickness of 0.20mm. The capillaries were charged with the enzyme sol using capillary action (see figure 6).

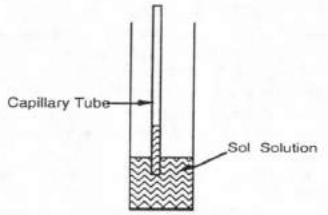

Figure 6. "Loading" of capillary with sol.

The sols rose an average of 10 mm in the capillary tubes. Final test preparations of the gel in the capillary tubes were the same as described above. The dried gels were transparent and the enzyme maintained high activity as evidenced by visual studies. However, a severe limitation of using the capillary tube was that the dried gel did not coat the tubes' inner surfaces evenly, but instead formed plugs at the bottom of the capillary tube. This plug prevented efficient delivery of the analyte to the gel test system (see figure 7).

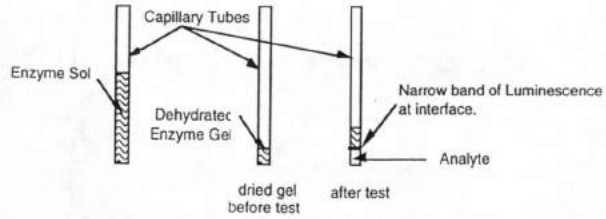

Figure 7. Formation of gel "plug" in capillary tube.

Coenzyme A, sodium salt from yeast, was purchased from Sigma. This co-factor acts to moderate the luciferase turnover rate and avoid an initial peak output, providing a longer, albeit lower intensity steady-state luminescence. This prolonged output is more suitable to the device.

Potato apyrase was purchased from Sigma. The apyrase solution was prepared with 0.45M GlycylGlycine, pH 7.8. The apyrase dephosphorylates ATP.

$$ATP \xrightarrow{\text{apyrase}} ADP$$

Trehalose was purchased from Sigma.

Adenosine Triphosphate (ATP), disodium salt was purchased from Sigma.

$MgSO_4$ was purchased from Sigma. The magnesium ion, along with molecular oxygen, luciferase, luciferin, and ATP, is required for luminescence.

$$Mg^{2+} + ATP + luciferin + O_2 \xrightarrow{\text{firefly luciferase}} AMP + oxyluciferin + light + pyrophosphate$$

Two different agarose gel compositions were explored. Low melting temperature SeaPlaque agarose, gelling temperature 30°C, was purchased from FMC Bioproducts, and super low melting temperature agarose, S.LM3, gelling temperature 18°C from Hispanagar was used.

Luciferase, apyrase, coenzyme A, luciferin, and $MgSO_4$ were mixed and entrapped in the agarose gels. Both gel types (low temperature and super low temperature) were prepared with 0.45M GlycylGlycine buffer, pH 7.8 to a final concentration of 1% (w/w) solution. The gel solutions (in the sol state) were heated to 80°C and then cooled to 37°C for the SeaPlaque agarose, and cooled to 25°C for the Hispanagar agarose. After cooling, luciferase, apyrase, coenzyme A, luciferin, and $MgSO_4$ solutions were added to the sol containing 100mg/ml (final concentration) trehalose.

Six combinations of the reaction components were tested for stability.

luciferase	luciferase, luciferin
luciferase, apyrase	luciferase, coenzyme A
luciferase, luciferin, coenzyme A	luciferase, luciferin, coenzyme A, apyrase

The sols were placed at 4°C for one hour to ensure gelation, then air dried at room temperature for 6 to 48 hours and stored in an air tight container at 4°C until testing.

Enzyme activity was measured with a CCD camera and a PMT. Visual and instant photographic records were also made.

In some cases we rehydrated the gels prior to addition of the analyte solution. In another set of experiments we used the analyte solution itself as the rehydration fluid. In each case, the analyte contained the reaction components not already in the gel as well as a range of concentrations of ATP.

We determined that a 1% (w/w), super low gelling point agarose gel (Hispanagar S.LM3), with trehalose provided the best support for long term enzyme storage. Optimum stabilization was obtained when the sol was cooled to 4°C to induce gelling, dried at 22°C and 20% relative humidity for 6 hours and stored in an air sealed container at 4°C. Storage times up to six months for both luciferase (native and recombinant) and apyrase were studied with both luciferase and apyrase maintaining at least 50% activity. Storage of the luciferin substrate and coenzyme A in dehydrated gels was not as successful. However, the lyophilized powder of these components maintain their activity indefinitely when stored at -20°C, and we propose adding these as fresh components of the test solution (analyte). One of the phase II tasks is to enhance the stability of luciferin in dehydrated gels.

Results: Luciferase and apyrase can be preserved, maintaining at least 50% activity, in a dehydrated state for at least 6 months under the proper preparation and storage conditions, described above.

The cotton fiber studied was a "100% Cotton" embroidery floss. The fibers were charged with the enzyme sol by immersing the fiber in the sol. The sols coated the fibers thoroughly and was then allowed to gel. Final test preparations of the gel on the cotton fiber were the same as those described above. The dried gels demonstrated a greatly reduced enzyme activity as evidenced by visual observation. The reason for the reduced enzyme activity was caused by the wicking properties of the cotton fiber. The gel phase and the liquid phase containing the enzyme were separated due to this wicking. With the gel structure no longer protecting the enzyme from exposure to an air liquid interface, the enzyme activity decreased.

The filter paper studied was Whatman No. 1. The paper was charged with 50 µl of the enzyme sol by application with a manual pipet. The filter paper performed in much the same way as the cotton fiber. The agarose portion of the liquid solution remained where it was deposited on the filter paper, but the liquid component, including the proteins, was wicked away from the deposit site, exposing the proteins to the paper and an air interface. See figure 8. Upon air drying the gel, the proteins remained in the liquid phase, separated from the gel. No enzyme activity was detected visually. The reason for the decline in activity was for the same reasons stated above for the cotton fiber.

Figure 8. Phase separation in filter paper.

The polyester film and polystyrene plates provided the best support for the gel. The gel did not wet the glass tubes, forming a "plug" which prevented delivery of additional substrates. The experiments which dealt with the cotton fiber and the filter paper as supports showed that the gel wicked along these supports in such a way as to allow convenient application of substrate. However, the air interface to which the proteins were exposed compromised their stability. For this reason, we chose both the film and plates as suitable supports for the protein gel films.

Results: The nonporous structures of agarose coated polyester film, the polystryrene titration plates and glass capillary tubes provided the best support for the enzyme gels.

4. Capillarity–Sample Delivery. *Objective: To develop a delivery system which ensured uniform and fast delivery of the analyte to the test spots.*

As discussed for gel spreading, the capillarity studies included experiments with polyester film, glass capillary tubes, cotton threads, and filter paper.

By incorporating the best features of the supports studied in objective 3, we examined two methods of sample delivery via capillarity and wicking. These methods were based on the suitability of both the polyester film and capillary tubes as protein gel supports and the excellent wicking characteristics of both the filter paper and the cotton fiber. One design incorporated two polyester films precoated with the dried enzyme gel sandwiched around a filter paper for analyte delivery. The other design incorporated a cotton fiber threaded through a capillary tube. See figure 9.

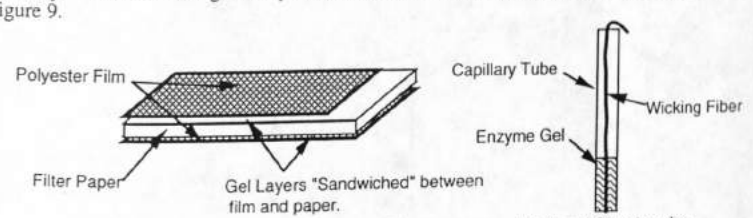

Figure 9. Two possible device designs, filter paper sandwich and fiber in tube.

The procedures for building these devices are discussed in detail in objective 7, Device Design and Fabrication.

To test the sample delivery rate of these designs and record their ability to rehydrate the dehydrated gels, simple tests of luminescence were undertaken. An analyte solution containing ATP, luciferin, coenzyme A and $MgSO_4$ were delivered to the dried enzyme gel, in the respective device. The analyte was delivered to the filter paper sandwich at the "open" end of the support, and the analyte was delivered to the cotton fiber tube at the lower end, near the gel. Delivery consisted of placing the respective ends in a shallow tray of the analyte and allowing delivery by capillary and wicking action.

The devices were observed for luminescence over a period of several minutes. Photographic records were kept. Uniform delivery to the entire filter paper sandwich occurred within two seconds and within 5 seconds for the fiber tube device. Both devices reached a steady-state luminescence after three minutes exposure to the analyte solution.

The benefit of these designs is that they provide a suitable physical support for the protein gel allowing the stable preservation of the protein while providing a mechanism for fast and simple sample delivery to the gel. They take advantage of the better stabilization character of the film and capillary tubes while utilizing the excellent wicking properties of the filter paper and fiber.

Results: A combination of fiber/filter wicking, and plate or tube capillarity will deliver the analyte to the test site quickly and uniformly.

5. Simulation of Capillarity–Gel Diffusion–Enzyme Reaction Rates. *Objective: To determine optimum enzyme concentrations and test strip geometry to ensure reliable and repeatable analyte testing.*

Tests involving the capillary delivery of ATP (see objective 4) showed that the delivery rates by capillarity were fast enough to deliver the analyte to all gel positions (an area 1 cm by 5 cm) within two seconds. This is a rate two orders of magnitude faster than the rehydration time of the gels which required up to 2.5 minutes for a gel volume of 50µl. Also, evaluation of the relative reaction rates of apyrase and luciferase showed that the apyrase turnover rate was over 100 times faster than that of luciferase (see objective 1). The fast capillarity and enzyme reaction rate are features which ensures quick consumption of excess ATP and a rapid readout.

Results: These successful experiments allowed optimization of apyrase/luciferase concentrations and test strip geometry through test evaluation rather than computer simulation.

6. Gel Enzyme Patterning/Printing. *Objective: To determine the optimum gel pattern for analyte concentration measurement.*

Review of literature on human vision revealed that at reduced light levels, humans can only resolve individual spots which are separated by several degrees of arc (23-25). Because resolution of the minimum required separation distance can be achieved using manual deposition of gel spots on the support, we did not require higher resolution printing technologies. Three test configurations were chosen for further analysis. Our patterns consisted of a series of 6 spots on the clear polyester film, 6 adjacent wells in an opaque white microtitration plate, and 6 cotton fibers in capillary tubes which were placed side by side. Each spot or fiber had the same gel (a 1% super low gelling point agarose with 100 mg/ml trehalose) and the same concentration of luciferase (0.1 mg/ml) but a varying concentration of apyrase (from 0 to 5 units/ml). Tests for pattern recognition were run using an analyte solution (luciferin at 0.5 mM, CoA at 0.5 mM, and $MgSO_4$ at 10 mM). A range of ATP concentrations from 10^{-3} to 10^{-9} molar were assayed. Light intensity was recorded using the CCD camera; instant photographic (Polaroid 3000 speed, 1 minute exposure) and visual observation records were also kept.

The dehydrated samples were exposed to the analyte solutions for rehydration. As the devices were exposed to the analyte, timed records of light intensity were kept. It was found that three minutes was required for the devices to reach an equilibrium luminescence.

Through variation of the specified apyrase concentration within the given ATP concentration range, the apyrase concentration gradients were optimized for specific ranges of

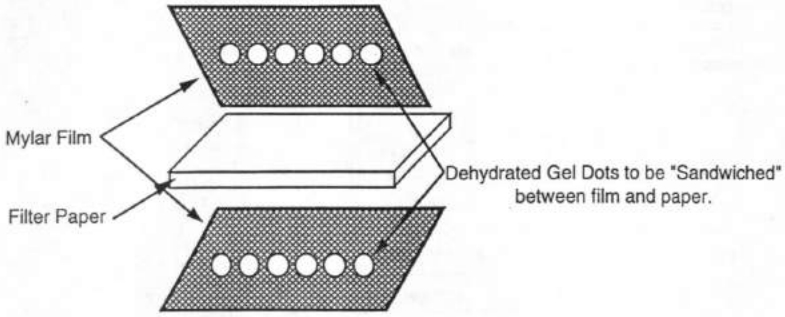

Figure 11. Assembly of filter paper sandwich device.

The wicking capillary tubes were made by first threading the cotton through the capillary tube and securing one end to avoid pulling the fiber out. The other end of the thread was trimmed to ease delivery of the gel. The gel was delivered into the capillary tube by simply dipping the trimmed end into the enzyme sol and allowing capillarity and wicking to pull the sol into the tube. See figure 12.

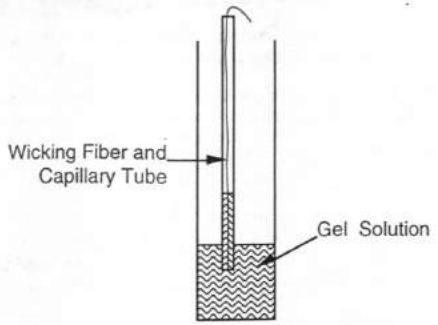

Figure 12. Construction of fiber in tube device.

Results: Two designs are promising. The first device involves a filter paper sandwiched between two gel coated film supports. The second device involves a cotton fiber wick inside a gel coated capillary tube.

8. Optical Imaging. *Objective: To evaluate the prototype spatial detection devices through bioluminescent intensity.*

The gels were imaged with three devices: a CCD camera, a Polaroid camera and visually. Because we designed our device to demonstrate ATP concentration with an on/off light output, our imaging tests were centered around the detectability of light by the respective imaging system.

ATP. pH paper test strips are designed and produce in an analogous manner; separate test strips are employed depending on the range and accuracy required. See figure 10.

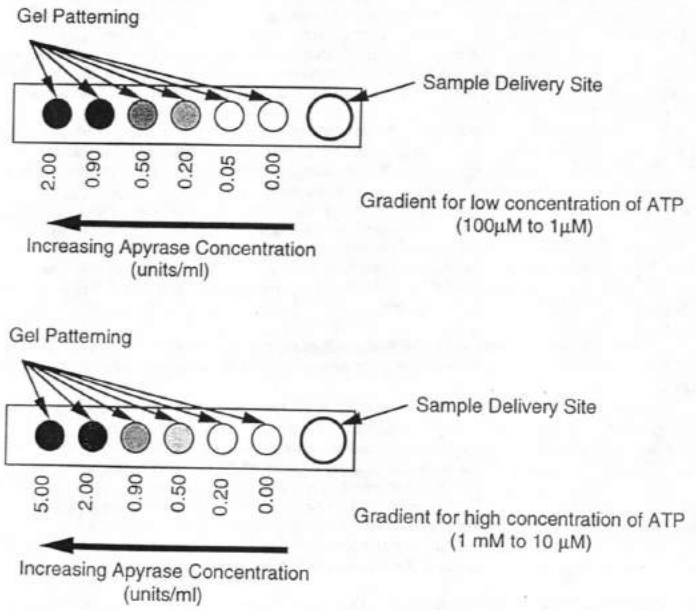

Figure 10. Different apyrase gradients for measuring different ranges of ATP concentration.

Levels of ATP down to 10^{-9} M could be measured using the CCD camera, but concentrations below 10^{-6} M could not be recorded either visually or photographically.

Results: Macroscopic deposition of gel dots allows optimum pattern recognition in low light conditions.

7. Device Design and Fabrication. *Objectives: To compile data from gel enzyme activity, capillarity and rehydration studies and design a spatial detection device .*

As described earlier, the two proposed device designs incorporate the beneficial features of a suitable support structure (film or capillary tube) and the excellent wicking features of either the fiber or the filter paper to allow fast delivery of the analyte solution.

The filter paper sandwich device is made by depositing two rows of enzyme gel dots (50μl) with their respective concentrations of apyrase (see gradients in figure 2) on the film support. Once the gels are dehydrated, they are pressed into the filter paper, thus adhering to both the film and the filter paper, without wicking away the liquid phase as experienced when the sol was applied to the filter paper. To enhance light output, the filter paper is sandwiched between two such film supports allowing double the gel volume to be utilized. See figure 11.

Generic test plates were developed for imaging. They incorporated a two dimensional gradient. In one direction we varied the concentration of apyrase. In the second direction, we varied the concentration of ATP in the analyte. Each of the dots had identical amounts of luciferase. Total number of gel spots on each plate was 36. The images of these plates were recorded using the CCD camera, the Polaroid camera, and the eye. See figure 13.

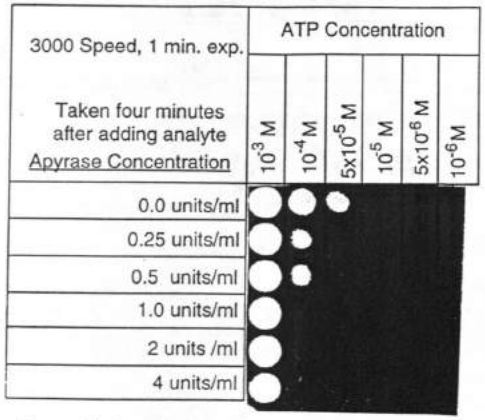

Figure 13. Reproduction of instant photographic record of ATP/Apyrase gradient in microtitration plate.

Observation of the test plates began immediately after infusing the dehydrated gel with the analyte. Because the light output is both concentration and time dependent, a rapid delivery system was designed using a six channel pipet. Records were kept for ten minutes following analyte delivery (samples reached equilibrium light emission in three minutes). To measure how different individuals would respond, groups of up to five people were asked to observe and record the luminescence from a single plate. Prior to observation, the people involved were kept in a dark room from zero to five minutes to measure the effect of dark adaptation on luminescent observation. For concentrations down to 10^{-6} M ATP, the major light inflection point was accurately recorded by the individuals involved. The instant Polaroid photography met with similar results. The CCD camera was able to record major inflections down to concentrations of 10^{-9} M ATP.

Results: Each device design demonstrated the ability to measure ATP concentration based on spatial position of light intensity. Optimization of the system to increase light intensity will improve both visual and photographic detection methods.

9. Device Testing. *Objective: To evaluate the device practicality from an ease of use and storage standpoints.*

The enzyme stability of the prototype device designs were tested for a variety of storage conditions. Test plates with enzyme gels (0.1mg/ml luciferase and varying concentrations of apyrase in 1% super low gelling point agarose with 100 mg/ml trehalose) were stored in three temperatures (-20°C, 4°C and 20°C) for up to 6 months. A range of ATP concentrations were studied from the standpoint of detectability and reproducibility.

Activity was measured using a CCD camera image of the microtitration plates. Fresh assay solution and ATP solutions were prepared for each series of tests.

Enzyme activity (luminescence) decreased by an average of 50% over a six month storage time with most of the loss coming in the first month of storage. However, since detectability depends on the relative activity of the luciferase and apyrase, no loss in overall detectability was suffered.

Results: ATP concentration detectablity does not decrease with long term (6 month) storage. The device, as configured, is relatively easy to use.

10. "Front end" enzymes–recommendations. *Objective: To assess the feasiblity of incorporating other ATP-dependent enzymes into the spatial detection device for concentration analysis of other substrates.*

This spatial detection method can be extended to the detection of a wide range of biochemicals and substrates by use of additional substrate specific enzymes dependent on ATP concentration for their activity. One example of this is the detection of hexose substrate concentration based on the enzyme hexokinase. Hexokinase is an enzyme which consumes ATP to transform glucose to glucose-6-phosphate. Because hexokinase is a fast enzyme compared to luciferase, it possible to use hexokinase as a front end enzyme; allowing our spatial detection system to measure glucose concentration in an analyte.

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

Preliminary tests of this system were run as follows.

Hexokinase solutions (20 units/ml) were prepared. Glucose solutions (10 mg/ml) were prepared. Luciferase solutions (0.4 mg/ml) were prepared. Solutions of ATP (0.1 mM) and stock assay solutions of 0.5 mM luciferin, 0.5 mM coenzyme A and 10 mM $MgSO_4$ were prepared.

Tests were run on two solution systems, one type containing all components (hexokinase with glucose plus the luciferase, ATP and assay), the other without hexokinase. Using a PMT to measure relative light output, we recorded a rapid decrease in luminescence after 10 seconds for the sample containing the hexokinase, see figure 14.

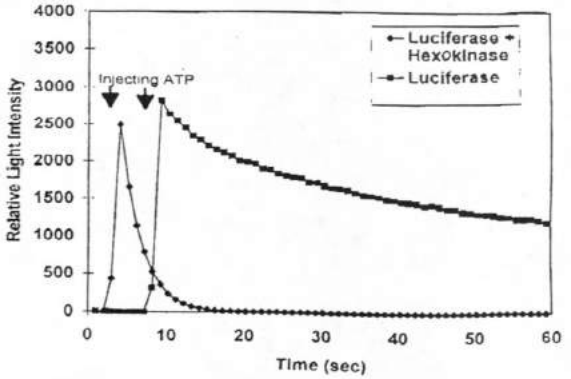

Figure 14. Light intensity output of hexokinase and "plain" control test system.

Results: It is feasible to incorporate hexokinase, or a similar enzyme system, into the spatial detection device to measure substrate (glucose) concentration.

Firefly luciferase-based bioluminescence reactions are now widely used for the detection of the presence of bacteria. These ATP detection methods are also used for "hygiene monitoring", to insure good laboratory and manufacturing practices, thereby minimizing bacterial inputs at all stages of food manufacturing and preparation processes. A number of companies supply the reagents and equipment for these applications, including Bio-Orbit (Turku, Finland), Lumac (Landgraaf, The Netherlands), Analytical Luminescence Labs (San Diego, CA), Los Alamos Diagnostics (New Mexico), Charm Sciences (Malden, MA) and Foss Food Technology, Inc. (Eden Prairie, MN). Companies who market more conventional bacteria culture and colony counting equipment (3M Corp. for example) are eager to move into the area of rapid microbiological analysis.

Our approach to ATP analysis obviates the need for luminometers or photon counters for low level ATP detection, thus making such analysis far more widely applicable than it is today. Several companies who employ firefly luciferase-based ATP detection to monitor for the presence of microbes have expressed interest in PSI's technology and we have entered into serious negotiations with one of these firms. We will continue to present this technology to interested corporations until a corporate partner is located. It is our intent to provide the technical support and research and development efforts necessary to develop a useful product. We then plan to license the technology, or provide OEM devices to our corporate partners who possess the necessary marketing skills and market presence to make the products a commercial success. A complete phase III development and commercialization plan will be submitted as part of the Phase II application.

In addition to applications for microbial detection, which would require increased sensitivity, the existing technology can be used for substrate detection other than ATP. In these applications, the depletion of ATP from a second enzyme reaction is quantified, and is proportional to the amount of the substrate present. The list of potential analytes includes glucose, galactose, and many other compounds, and can be expanded as front-end enzymes are employed. Similarly, the technology is applicable to the detection of enzymes which consume, or liberate, ATP. Again, by employing appropriate substrates, the list of possible analytes is large. These applications (detection of specific substrates and enzymes) will be the subject of future phase I proposals.

TECHNICAL FEASIBILITY

For applications in the detection of microorganisms, the area with the greatest market potential and the subject of our phase II proposal, sensitivity of the assay must be improved from 10^{-6} M to 10^{-9} M ATP. Preliminary studies have indicated that the use of high-speed film to integrate the emitted light over a period of exposure of one to several minutes can increase sensitivity by up to 2 orders of magnitude. We are just beginning to experiment with the use of waveguides and other optical means of focusing the emitted light onto the photographic film (20,000 ASA), and anticipate an increase in sensitivity of at least one order of magnitude by incorporating waveguides and optical focusing. Thus sensitivity can be improved to detect ATP in nanomolar concentrations.

More complex, yet promising, technology is the incorporation of enzyme amplification schemes into the final device design. In these systems AMP, a byproduct of the luciferin-luciferase bioluminescent reaction, is phosphorylated to ATP by an appropriate enzyme system (29). The ATP can then again interact to emit light. Thus, prolonged light emission can be monitored via film detection.

All of these methods – photographic film detection, optical focusing and waveguiding, and enzyme amplification – will be addressed in the phase II studies.

For analysis of substrates or enzymes that interact with ATP, no increase in sensitivity is needed. However, the suitable ATP-ases must be selected and the interactions between the analytes and the detection system must be determined in order to optimize the system so that the maximum ATP change can be realized. These applications will be the subject of future phase I studies.

11. Bacterial Systems Recommendation. *Objective: To examine, in a preliminary manner, the possible use of Bacterial luciferase as a direct reading biosensor using our spatial separation technology.*

The bacterial luciferase system is more complicated than the firefly luciferase system, requiring two enzymes and the minimization of oxygen-dependent side reactions:

$$(1)\quad \text{NADH or NAD(P)H} + \text{FMN} \xrightarrow{\text{oxidoreductase}} \text{FMNH}_2 + \text{NAD} + \ldots.$$

$$(2)\quad \text{FMNH}_2 + \text{Aldehyde} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{FMN} + \ldots.. + \text{light}$$

$FMNH_2$ is unstable, reacting with oxygen to form FMN and peroxide. Thus most application of bacterial luciferase for biosensors and other analytical purposes utilize the 2 enzymes in close proximity – possibly in direct contact or as a weak complex (26-28). In this case the overall reaction is

$$\text{NADH or NAD(P)H} + \text{aldehyde} + \text{O}_2 \xrightarrow[\text{luciferase}]{\text{oxidoreductase,}} \text{NAD} + \ldots. + \text{light}$$

We must deliver aldehyde to the reaction. The aldehyde is also unstable, oxidizing under many conditions in 8-10 hours. Thus it is desirable to produce the aldehyde directly, enzymatically, from a suitable precursor. Marine bacterial enzymes are certainly available for this purpose, but we have not yet examined their practicality for our purposes.

Development of a quantitative NADH or NAD(P)H sensor via spatial analysis requires an NADH or NAD(P)H "consumase", analogous to the ATP "consumase" (apyrase) discussed earlier. There are some possibilities utilizing lactic acid and/or ethanol synthesis enzymatic reactions.

Results: The bacterial system is sufficiently complicated that it is not appropriate to pursue it as part of this project.

FINAL COMMENTS

A device for measuring the concentration of ATP, using either human vision or Polaroid photography, was successfully designed, built and tested at Protein Solutions, Inc. in cooperation with the University of Utah. The current sensitivity of the device is in the micromolar range using either visual recording or a short exposure 3000 speed film. We expect that this sensitivity can be enhanced by three orders of magnitude (nanomolar range) without major device redesign. This enhancement will be the result of 1) a ten minute exposure, 2) using 20,000 speed film, 3) and utilization of the waveguiding properties of the gel "sandwich". It is important to note that our device does not require specialized equipment or the special training required to operate such equipment. With further refinement of enzyme/substrate concentrations, light focusing techniques, and ATP amplification (29), it is expected that picomolar range measurements will be achieved.

COMMERCIALIZATION PLAN

The initial market for direct reading ATP sensors is the food and dairy industry, agencies responsible for the safety and monitoring of food and dairy products, and the consumer concerned with food safety.

There has been growing interest in the last several years in the development and application of very rapid and simple methods for the monitoring of microbial contamination of food (30,31). There is also growing interest and concern over the apparently growing incidence of bacterial infection due to common organisms and antibiotic-resistant organisms (32).

Stability of the enzymes is always an issue. We have successfully improved stability by incorporating luciferase into agarose gels and drying the gels under controlled conditions with suitable additives. Trehalose has been successfully employed as an additive thus far, yet many other more readily obtainable additives (i.e. sucrose) may prove to be equally effective stabilizers. These will be studied in phase II.

Configuration of the elements of the device into an affordable product should be relatively straightforward. We plan to rely on much of the technology developed for dry reagent chemistry applications. Partnering with a corporation with manufacturing experience in these applications is anticipated, and should greatly facilitate the development of the technology into an manufacturable product.

Cited References:

1. K. Wood, Ph.D. Dissertation, Department of Chemistry, University of California, San Diego, 1989.
2. J.W. Thorner and H. Paulus, "Catalytic and Allosteric Properties of Glycerol Kinase from *E. coli*," *J. Biological Chem.* **248** (1973) 3922-3932.
3. The Kodak Ekta-Chem clinical chemistry tabs are a good example.
4. L.J. Kricka, et al., *Anal. Applic. of Bioluminescence and Chemiluminescence*, Acad. Press., 1989.
5. T.O. Baldwin, et al., "...Immunoassay...Using Bacterial Luciferase," *Meth.Enzym.* **133** (1986) 248-264.
6. D. Griffiths and G. Hall, "Biosensors -- What Real Progress is Being Made?," *TIBTech* **11** (1993) 122-130.
7. A. Lundin, "ATP Assays in Routine Microbiology: From Visions to Realities in the 1980's," in *ATP Luminescence, Rapid Methods in Microbiology*, P.E. Stanley, B.J. McCarthy, and R. Smither, eds., Blackwell Scientific, 1989, pp. 11-30.
8. C.Y. Wang and J.D. Andrade, "Interfacial Behavior in Firefly Luciferase," in A.A. Szalay, et al., eds., *Bioluminescence and Chemiluminescence: Status Report*, Wiley, 1993, pp. 99-103.
9. P.E. Stanley, "A Concise Beginner's Guide to Rapid Microbiology Using Adensoine Triphosphate (ATP) and Luminescence," in *ATP Luminescence, Rapid Methods in Microbiology*, P.E. Stanley, B.J. McCarthy, and R. Smither, eds., Blackwell Scientific, 1989, pp.1-10.
10. L.J. Blum, et al., "Design of Luminescence Photobiosensors," *J. Biolum. Chemilum.* **4** (1989) 543-550.
11. P.R. Coulet, "Luciferase-based Sensors," in Proc. Second World Congress on Biosensors, *Biosensors 92*, Elsevier Adv. Tech., pp. 2-9.
12. P.J. Worsfold and A. Nabi, "Bioluminescent Assays...," *Anal. Chim.Acta* **179** (1986) 307.
13. A.Nabi and P.J. Worsfold, "Determination of Ethanol and Alcohol Dehydrogenase Using Co-immobilised Bacterial Luciferase and Oxidoreductase," *Analyst* **111** (1986) 531-533.
14. A. Roda, et al., "Coupled Reactions for the Determination of Analytes and Enzymes Based on ... Luminescence," *J. Biolum. Chemilum.* **4** (1989) 423-435.
15. F.H. Harold, *The Vital Force: Bioenergetics*, W.H. Freeman, Co., 1986.
16. U. Wollenberger, et al., "Biosensor for ADP...," *Anal. Lett.* **20** (1987) 657-668.
17. U. Wollenberger, et al., "Enhancing Biosensor Performance Using Multienzyme Systems," *TIBTech* **11** (1993) 255-262.
18. C.Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and L. Kricka, eds. Wiley, 427-432 (1991).
19. C.Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 423-426 (1994).
20. D.J. Min, C.Y. Wang and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Cambell, L.J. Kricka, and P.E. Stanley eds. Wiley, 423-426 (1994).
21. C.Y. Wang, R. Stewart and J.D. Andrade, "Purification, Stability and Applications of Biotinylated Recombinant Luciferase", in preparation (1995).
22. Chung-Yih Wang, Ph.D. Dissertation in preparation, "Firefly Luciferase at Model Interfaces: Activity, Stability and Sensor Application", expected December 1995.
23. R.F. Hess, et al. eds. *Night Vision*, Cambridge University Press: UK (1990).
24. M.H. Pirenne and E.J. Denton, "Accuracy and Sensitivity of the Eye," *Nature* **170** (1952) 1039.
25. A. Rose, "Quantum effects in Human Vision," *Adv. Biol. Med. Physics* **5** (1957) 211.

For exameple, B.H. Schneider, et al., "Microminiature Enzyme Sensors for Glucose and Lactate Based on Oxygen Electrodes," *Sensors and Actuators*, **B1** (1991) 565-570.

N.N. Ugarova, et al., "Bioluminescent Microassay of Various Metabolites Using Bacterial Luciferase Co-immobilized with Multienzyme Systems," *Analytical Biochemistry* **173** (1988) 221-227.

J.D. Andrade, ed., *Medical and Biological Engineering in the Future of Health Care*, U. of Utah Press, 1994, see chapters by I. Karube and J. Andrade.

J.D. Andrade, R.J. Scheer, "Applying 'Intelligent Materials for Materials Education: The Lableess Lab™," *Proc., 2nd Annual Conference on Intelligent Materials*, Tech. Publ. Co., 1994, in press.

J.D. Andrade, et al., "Proteins at Interfaces: Principles, Problems, and Potential," chapter in *Interfacial Behavior of Bioproducts*, J. Brash and P. Wojciechowski, eds., Dekkar, 1994, in press.

J.D. Andrade, et al., "Using Bioluminescence for Integrated Science Education," in A.A. Szalay, et al., eds., *Bioluminescence and Chemiluminescence: Status Report*, Wiley, 1993, pp. 69-73.

26. S.M. Gautier, et al., "Bioluminescence-Based Fibre-Optic Sensor With Entrapped Co-Reactant...," *Analytica Chimica Acta* **243** (1991) 149-156.
27. S.M. Gautier, P.E. Michel and L.J. Blum, "Reagentless Bioluminescent Sensor for NADH," *Anal. Lett.* **27** (11) (1994) 2055-69.
28. K. Oda, S. Yoshida, S. Hirose, T. Takeda, "Ultratrace Levels of ... NADH by use of Immobilized Luciferase," *Chem. Pharm. Bull.* **32** (1984) 185-192.
29. D. Pfeiffer, et al., "Cascade-like Exponential Substrate Amplification in Enzyme Sensors," *Biosensors & Bioelectronics* **10** (1995) 169-180.
30. D.Y.C. Fung, "Rapid Methods and Automation in Food Microbiology", *Food Reviews Int.* **10** (3) (1994) 357.
31. D.Y.C. Fung, "What's Needed in Rapid Detection in Foodborne Pathogens," *Food Tech.* June (1995) 64.
32. J.Y. D'Aoust, "Salmonella and the International Food Trade", *Int. J. Food Microbiol.* **24** (1994) 11.

Bibliography

J.D. Andrade, et al., "Immuno-Biosensors: The Clinical Chemistry and Coagulation Laboratory on a Chip," in Y. Sezai, ed., *Artificial Heart: Biomation in the 21st Century*, Saunders, 1992, pp. 89.
J. Luo, M.S. Thesis, U. of Utah, in preparation.
"Handbook of ATP-Hygiene Monitoring", Bio-Orbit, Turku, Finland.
Special issue, "Bioluminescence in the Sea," *Naval Research Reviews* **45** (1993) (#2).
J. Lee, "Bioluminescence: Biochemistry for Fun and Profit," in D.P. Valenzeno, ed., *Photobiological Techniques*, Plenum, 1991, pp. 297-321.
T. Arakawa, S.J. Prestelski, W.C. Kenney, and J.F. Carpenter, "Factors Affecting Short-Term and Long-Term Stabilities of Proteins," *Adv. Drug Delivery Reviews* (1993), in Press.
J.F. Carpenter and J.H. Crowe, "Modes of Stabilization of a Protein by Organic Solutes During Desiccation," *Cryobiology* **25** (1988) 459.
J.H. Crowe, L.M. Crowe, D. Chapman, "Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose," *Science* **223** (1984) 701.
C. Colaco, et al., "Extraordinary Stability of Enzymes Dried in Trehalose," *BioTech.* **10** (1992) 1007.
B.P. Gaber, et al., "Interaction of Trehalose..." Molecular Modeling," in A.C. Leopold, ed., *Membranes, Metabolism, and Dry Organisms*, Cornell Univ. Press, pp. 231.
J.H. Crowe, et al., "Anhydrobiosis," *Ann. Rev. Physiol.* **54** (1992) 579.
A.F. Dukhovich, N.N. Ugarova, and I.V. Berezin, "Firefly Luciferase as a Complex of Protein with Lipids," *Dokl. Acad. Nauk SSSR* **289** (1986) 231-233.
V. Kratysuk, Abstract, *Conference on Bioluminescence*, Maui, Dec., 1993.
J.D. Andrade, C.Y. Wang, V. Hlady, P. Triolo and R.J. Scheer, "Method of Measuring Chemical Concentration and/or Light Intensity Based on Spatial Separation and Resolution", Preliminary US Patent Application (*1995).
N.N. Ugarova and O.V. Lebedeva, "Immobilized Bacterial Luciferase..." *Appl Biochem Biotech* **15** (1987) 35-51.
J.W. Hastings, "Bioluminescence in Bacteria and Dinoflagellates," in *Light Emission by Plants and Bacteria*, Acad. Press, 1986, pp. 363-398.
I. Bronstein and A. Sparks, "...Enzyme Immunoassays With Chemilum...," in R.M. Nakamura, et al., eds., *Immunochemical Assays and Biosensor Technology*, Amer. Soc. Microbiol., 1992. pp. 229-250.
R.F. Zuk, et al., "Enzyme Immunochromatography," *Clin. Chem.* **31** (1985) 1144-1150.
A. Nabi and P.J. Worsfold, "...Assays...Using Immobilized Bacterial Luciferase," *Analytical Letters*, **22** (1989) 1861-1871.

PROPRIETARY INFORMATION:

From page 6.

The E. coli strain BL21 DE3 was transformed with plasmid pRSET-BCCP-Luc and cultured in Luria broth (LB) containing 50 µg/ml ampicillin at 37°C. After a 100 fold dilution with fresh LB (6 hours) followed by 6 additional hours of growth, the cells were harvested by centrifuge (7000rpm at 4°C for 20 minutes). The cell pellet was resuspended and washed in Tris buffer (50 mM, pH 7.8). The cells were treated with a protease inhibitor (1 mM phenylmethysulfonyl fluoride, PMSF, in isopropanol) and disintegrated by sonication. The cell lysate was centrifuged at 15,000 rpm and 4°C for 25 minutes, and the supernatant containing recombinant luciferase was collected.

Ni-NTA agarose (purchased from Qiagen) is washed with 10 ml Tris buffer and packed into a column. The column is equilibrated with 10 ml Tris buffer. 2 ml of supernatant is applied to the column. The histidine tagged luciferase binds specifically to the Ni^{2+} chelate agarose. Two aliquots of 2 ml Tris buffer is used to elute the unbound proteins. Imidazole, which competes with histidine for coordination sites of the nickel ion, is used as eluent. The eluent containing the purified luciferase is collected.