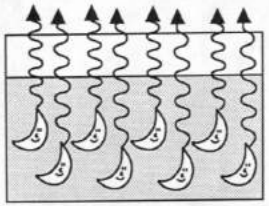
Title: **Living Optical Materials: The Bio-Photon Project**



A Pre-Proposal/White Paper to

To: MURI Program FY95

Topic: 7. Advanced Biosensor Arrays
Topic: 8. Advanced Optical Materials
Topic: 10. Materials and Processing at the Nanomater Scale

From: Center for Biopolymers at Interfaces and
Department of Biongineering
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Duration: 3 years.

Budget: ~ $1.0 M/year Total Costs

Corporate Collaborators (Tentative):

Matech, Inc., Westlake Village, CA
Protein Solutions, Inc., Salt Lake City, UT
3M Corporation, St. Paul, MN
Promega Corp., Madison, WI

Contents:

I. Objectives:

• *Photo-proteins.* To produce and study ordered 2-dimensional and 3-dimensional arrays of photo-proteins, particularly firefly, bacterial, and dinoflagellate luciferases. The goals are to control their orientation, stability, microenvironment, and activity, with the longer range goal of providing efficient light emitting surfaces and films for a range of applications.

Isolate the individual enzymes in controlled micro-environments which will facilitate their function as nanoparticles and possibly as quantum dots. Ordering processes would also include means to provide geometries which might be appropriate to quantum whiskers and other quantum reactive geometries.

Existing work on the stabilization and enhancement of photoenzyme properties in organic gels would be extended to include inorganic gels and dual enzyme immobilization for biosensor applications.

• *Bacteria.* To develop and produce artificial photo organs (biological flashlights) by the high density culture of marine bioluminescent bacteria. These living flashlights would be based on studies of the photo organs of bioluminescent fish and other marine organisms. The goal is to use existing biological knowledge to create an *in vitro* environment which would permit the development of artificial photo organs with useful brightness, longevity, and durability.

• *Dinoflagellates.* Dinoflagellates are relatively large marine phytoplankton, many of which are bioluminescent and photosynthetic. Some of these organisms are being maintained in closed cultures indefinitely. We want to study a possible artificial "symbiosis" between photosynthetic dinoflagellates and marine bioluminescent bacteria. The speculative goal is to develop an artificial photo organ utilizing the photosynthetic organism as the fundamental energy source and the bioluminescent organisms as the photon producers. Other possibilities are to use photosynthetic bacteria and bioluminescent bacteria. It is also possible to engineer a bacterial strain which would be both photosynthetic and bioluminescent, but such work is not proposed for this project at this time.

II. Rationale:

Although bio- and chemiluminescence are well known processes and are applied in a number of areas, they are generally considered to be impractical as photon sources, in part because the light produced tends to be non-oriented, non-polarized, of relatively low intensity, and to otherwise not have the more precise and well controlled characteristics of sources which utilize modern electro-optical materials.

The growing awareness and application of materials and devices with nano dimensions, and the importance of quantum phenomena in such devices, has led to the consideration of such quantum effects in bioluminescent systems. Although there is no evidence for this at the present time, it may be because bioluminescence processes have rarely, if ever, been approached from the perspective of quantum optics. Proteins and enzymes are colloidal particles of nano dimensions and may be capable of functioning as quantum dots. Proteins, and enzymes in particular, often have unique dielectric environments and geometries which can, at least in principle, influence the photon emission process, including both direction and polarization.

Firefly and bacteria luciferases are well characterized, partially understood, enzymes already widely applied in medicine, biochemistry, and biophysics as unique labels and markers. Several groups, including our own, are already using luciferases as components of molecular devices and biosensors.

On the cellular level, bioluminescent marine bacteria can be cultured at very high densities in unique photo organs to provide an intense, living bio-flashlight -- in a symbiotic relationship with the host fish. The host controls the environment in a manner which results in unusually dense cultures with particularly high photon intensities. In addition, such fish and other organisms have developed special optical components to enhance the light intensity and directionality, including lenses, filters, reflectors, wave-length shifters, and waveguides. It is time to take a hard look at these systems from materials science/engineering/optical physics perspectives.

Finally, some bioluminescent organisms are also photosynthetic -- in particular certain marine phytoplankton, the dinoflagellates. At least some of these organisms have much of their photon --

producing machinery packaged in membrane-connected organelles called scintillons. We have been culturing *Pyrocystis lunula*, an ubiquitous marine dinoflagellate, in closed plastic bag environments for several years. These high density, very bright cultures are being used for a wide range of Science in the Dark educational experiences and are now being developed for sensing and monitoring applications. Bacteria-dinoflagellate symbiosis is a relatively unprobed area of great scientific and possibly practical significance

A new Bio-Based Engineering program in our Department of Bioengineering now provides the infrastructure and resources with which to tackle a project on Living Optical Materials, hence this proposal.

III. Approach:

• *Photoproteins.* Our group works on proteins at interfaces and has had substantial experience with protein adsorption, immobilization, orientation, conformation and structural perturbation at interfaces. We have had experience in the modification of a wide range of surfaces, including gradient modification, and in the study and characterization of protein interactions with such surfaces. These include various optical surfaces suitable for interfacial spectroscopies, polymeric surfaces and materials suitable for medical devices and diagnostic products, and air/water interfaces, both as model systems for understanding interfacial processes and because of their importance in biotechnology and downstream processing.

In recent years we have developed expertise in micro and nano patterning of proteins on surfaces using photolithographic and related technologies. We would use these techniques and processes to produce and study 2 dimensional arrays of the various photo-proteins. We also have experience with protein/lipid, protein/protein, and protein/synthetic polymer interactions using dynamic surface tension and Langmuir Blodgett techniques. These techniques lend themselves to means of producing protein films of known orientation and with controlled microenvironments, including controlled dielectric characteristics.

Characterization methodologies include highly sensitive CCD camera based photon imaging systems, means to measure polarization, means to measure the spectral characteristics of photo emission, and means to measure lifetimes. We also have experience in energy transfer measurements and in protein/fluor systems.

• *Bacteria.* We routinely culture E-coli in which firefly luciferase has been expressed. This has been provided by our industrial collaborator, Promega. We are now considering the specific engineering of the luciferase in order to facilitate the purification process and orientation and its immobilization at interfaces.

We are also routinely growing marine bacteria. We are collaborating with the Inst. for Biophysics in Krasnoyarsk, Siberia (Russia) which has 2,000 different marine bacteria strains. We are, of course, interested in marine bacteria as a source of bacteria luciferase. We would also express the luciferase in E-coli, possibly in modified form for ease in purification and in subsequent orientation and fabrication.

Our major interest in marine bacteria is in the development of an artificial light organ using high density, oriented cultures of such bacteria. We have been examining in a preliminary way the role of the culture medium, the controlled delivery of nutrients, controlled removal of metabolic by products -- all with the goal of maintaining a high density culture indefinitely in a small volume. Indeed, one of our industrial collaborators, Protein Solutions, Inc. (PSI), is already attempting to develop a product for nicotine detection based on this small *in vitro* artificial photo organ and its inhibition by nicotine.

• *Dinoflagellates.* We have been working with bioluminescent dinoflagellates for the past four years and now have means of maintaining them in sealed bag culture for periods of up to three years, without feeding or addition of any nutrients. The bags are transparent low density polyethylene. These photosynthetic organisms receive light for photosynthesis through the bag and have some limited O_2/CO_2 exchange through the walls of the bag. Our industrial collaborator, PSI, already markets two products for science education based on this technology. These dinoflagellates have to be mechanically stimulated in

order to produce light, whereas the marine bacteria glow without such mechanical stimulation. We are therefore eager to develop a dual culture system, creating an artificial symbiosis between a photosynthetic organism and an organism with continuous light production characteristics to develop artificial photo organs which can exist for years as self-contained, miniature ecosystems.

Although it is unlikely that these "devices" would be competitive as high resolution displays, it is certainly not impossible. The goal at this stage is applications in the biosensor diagnostic arena, for environmental surveillance, emergency lighting, and related areas, although the possibility of a living, flat panel display is not entirely far fetched.

The cultures would be monitored by a range of analytical methods, including gas phase infrared and Raman spectroscopy, aqueous phase infrared, Raman and fluorescence spectroscopy, as well as through specific and semi-specific optically-based biosensors.

We have a large biosensor group and have been working on fiber-optic based fluoro-immunosensor systems for the past 15 years. We also have an active microelectronics group and a new program, as part of our Whitaker Bio-Based Engineering Program in the Department of Bioengineering, focused on micro- and nano-engineering, not only of silicon, but also of soft, polymeric, and biological structures. These activities are housed in a new Medical Biopolymers Building containing modern laboratory space. Two thirds to three quarters of the activities on this program would utilize that new facility.

Although oriented photoemission has been considered unlikely, the recent results in cavity quantum electro-dynamics suggest that the directionality of photon emission can indeed be predicted and controlled, given appropriate control of the nano structures and their dielectric environments. A probably outcome of this work may be oriented and at least partially polarized photo-emission from such unique thin films.

The work on oriented arrays is expected to tell us a great deal about the possible presence and role of nano structures in living bioluminescence organisms.

IV. People and Credentials

J. Andrade, P.I., is Professor of Bioengineering and Materials Science at the University of Utah. He is former Chairman of the Department of Bioengineering and former Dean of the College of Engineering. Joe has worked on proteins at interfaces and biocompatability, biosensors and related areas for 25 plus years, has edited 6 books, and has about 125 peer reviewed technical papers. He is experienced in the management of research organizations and research groups, has produced some 45 Ph.D. and Masters students, consults for a number of industries and government agencies, and has been the founder or co-founder of two companies, including Protein Solutions, Inc., one of the industrial collaborators in this project. He has been working on luciferases and bioluminescence for the last 4 years.

Dr. Vladimir Hlady, is a physical chemist/surface chemist/bioengineer on the faculty of the Department of Bioengineering. Vlado has worked extensively with biosensors, interfacial fluorescence, proteins at interfaces, and colloidal systems. He is particularly well recognized for his work on protein adsorption and total internal reflection fluorescence as applied to protein adsorption. His present work is focused on scanning force microscopy, protein patterning, and specific protein-ligand binding processes.

Dr. Russell Stewart, a new member of the Department of Bioengineering and its Bio-Based Engineering Program, is an expert on proteins involved in motility and transport and has a particular expertise in molecular biology and protein engineering. He is developing some of the micro and nano engineering laboratories in this new program together with Dr. Hlady.

The final proposal will include other faculty with a particular interest in electro and chemiluminescent systems and devices, molecular biology, biochemistry, and tissue engineering, including colleagues in the Departments of Physics, Chemistry, and Materials Science (Tresco, Xu, Cohen, Horch, Caldwell, Rapoport, Janatova).

Advisors and consultants will include J.W. Hastings, Professor of Biology at Harvard, one of the world's experts on bioluminescent bacteria and dinoflagellates; Professor James Morin, Professor of Biology at UCLA, one of the world's experts on fish photo-organs; Dr. Kenneth Nealson (not yet invited) with the University of Wisconsin in Milwaukee, another of the world's experts on bacterial bioluminescence; Dr. Peter Herring of the Institute of Oceanographic Sciences in England; and Dr. James Case of U.C. Santa Barbara. Dr.s Herring and Case are world renowned experts on bioluminescence and photo-organs. We would also expect to involve several of the leading individuals in the area of quantum dots, quantum whiskers, and cavity electrodynamics.

Our industrial collaborators include: Matech, Inc. in Westlake Village, CA, a high tech ceramics company with particular expertise in sol gel processing (Dr. Ed Pope). The 3 M Corporation, which has a major product line in reflective coatings and tapes and has a strong interest in self luminescing films (Dr.s A.P. Wei and James Vincent); Promega, Inc., Madison Wisconsin, a major biotechnology molecular biology firm which sells firefly luciferase and related products (Dr. Keith Wood); Protein Solutions, Inc. (PSI), a University of Utah spin-off company using bioluminescence for a variety of science educational kits, Science in the Dark product line. PSI has federal SBIR and STTR support for bioluminescence-based biosensors (Dr.s P. Triolo and R. Scheer).

Dr. Caldwell, Co-Investigator on this project, directs the Center for Biopolymers at Interfaces, a university/industry/state consortium of some twenty member companies focused on the behavior and application of proteins at interfaces. It is anticipated that most of CBI's members will have a major interest in this project. CBI's resources and facilities will be available to this project.

V. Budgets

Industrial Cost Sharing is yet to be determined, although it is expected that all four of the participant companies noted would provide resources, expertise, and facilities both directly and in kind to the project. The University of Utah will provide some release time for all of the major faculty participants to permit their involvement. Most of the faculty involvement is derived from the Departments of Bioengineering and Materials Science and Engineering. These are research intensive departments with relatively small undergraduate teaching commitments.

Undergraduate education will, however, be enhanced through the Center for Integrated Science Education (CISE) at the University of Utah, a project of the Colleges of Science, Engineering, and Education, and the University's Undergraduate Research Opportunities Program (UROP). J. Andrade, PI on this project, works closely with CISE and UROP and the project will involve undergraduates through CISE. We also expect to involve high school teachers as visiting scientists and assistants during the summer months.

We anticipate a final budget of roughly one million dollars per year (total costs), about $700,000 per year direct costs for *each* of the three plus two years which will be requested. Of the $700,000 about half would be used for faculty, post-doc, and student support, including benefits. About $150,000 would be requested for specialized equipment and facilities, about $25,000 for advisors, consultants, and industrial interactions, and the remainder split among the categories of travel, supplies, and special services such as analytical laboratories, device fabrication, shop expenses and publications. We we expect to involve 10 faculty,4 post-docs, 10 graduate students, and 10 undergraduates and/or high school teachers during each of the years of the project.

The University of Utah already has Technology Transfer agreements with Protein Solutions, Inc., and with the other members of the Center for Biopolymers at Interfaces. Promega and Matech are not yet members of this Center; appropriate technology transfer relationships would be developed and submitted with the formal proposal.

• *Further information*: contact J.D. Andrade, P.I., (801) 581-4379 phone, (801) 277-1259 home, (801) 585-5361 FAX, and e-mail: jdandrad@cc.utah.edu.

(1) [DOD-95- ↑]

Submitted to November 1994 program announcement:
FY95 DoD Multidisciplinary Research Program of the University Research Initiative

APPENDIX C: PROPOSAL COVER PAGE
(Both Sides of This Form Should be Completed and Submitted With the Proposal)

PROPOSAL NUMBER: _____
(To Be Completed by DoD Only)

1. THE PRINCIPAL INVESTIGATOR:

Dr.	Joseph	D.	Andrade	(801) 581-4379
(Title)	(FirstName)	(MI)	(Last Name)	(Phone Number, including Area Code)

University of Utah
(Organization)

Department of Bioengineering and Materials Science & Engineering
(Department/Division)

2480 MEB
(Street/P.O. Box)

Salt Lake City (City) Utah (State) 84112 (Zip Code)

CURRENT DoD CONTRACTOR OR GRANTEE: YES ____ NO X

If yes, give Agency, Point of Contact, Phone Number: _____

2. THE PROPOSAL:

Photons from Biology: The Biophoton Project
(Title; be brief and descriptive; do not use acronyms or mathematical or scientific notation)

8/1/95 - 7/31/2000
Proposed Research Period
(DD/MM/YY thru DD/MM/YY)

CEP 95
Your Institution's Proposal Number

OTHER AGENCIES RECEIVING THIS RESEARCH FUNDING REQUEST (e.g., NSF, DoE, NASA, NIH). Please identify agency(ies) and give Names(s) and Phone Number(s) of Point(s) of Contact at those agencies:

None

Submitted in Response to NRL (DoD Agency, e.g., ARO) Advanced Biosensor Arrays Advanced Optical Materials (DoD Agency research topic, e.g., Intelligent Turbine Engines)

Total funds requested from DoD:

$ 3,256,000	+	$ 2,045,000.00	=	$ 5,301,000
basic 3-yr total		2-year option total		5-year total

Abstract

Photons are basic to biology. Biology has learned to efficiently collect photons and transduce and utilize such energy via photosynthesis. Biology also utilizes photons for sensing and imaging purposes via vision. What is not as well known, and indeed has been largely ignored, particularly by the physics and engineering communities, is the fact that biology knows how to produce photons via bioluminescence.

Bioluminescence is an enzyme-based chemiluminescence process utilizing specific substrates (luciferins), oxygen, and often one or more co-factors. Although the photo-proteins involved have been used as labels in analytical chemistry, and as components of biosensors, there has been little or no interest in bioluminescence from the physics, materials science, or engineering communities.

We propose to develop ordered arrays of photoproteins and utilize these ordered arrays as optical microcavities and as components of optical devices. We address the question: "Does biology utilize microcavity optics?" If so, can we learn from this? If not, can we couple micro-cavity optical principles to bio- and chemi-luminescent-based devices?

The second major question: "Can man apply biology's unique bio-reactors?" refers to the fact that many fish and marine organisms have developed unique photo-organs, living flashlights. We propose to "develop" photoorgans based on highly dense, possibly ordered, cultures of marine bioluminescent bacteria. A variety of fish, such as the flashlight fish, have bright, highly efficient photoorgans. The unique micro-bioreactors used for the maintenance of these highly dense cultures should be studied from an engineering or materials science perspective.

We ask a third major question, "Can we develop a selective or artificial symbiosis between photosynthetic and non-photosynthetic bioluminescent organisms in order to develop and produce nearly infinitely rechargeable luminescent devices?"

The Bio-Photon Project thus consists of three major components:

- Photoproteins in Ordered Arrays;
- Artificial Photoorgans; and
- Artificial Symbiosis.

Potential applications include instrument-less biosensors, intelligent fibers and fabrics, living optical materials, including potential display applications. The project is thus responsive to four areas of the MURI initiative: functionally tailored textile fabrics, advanced bio-sensor arrays, advanced optical materials, and materials at the nanometer scale.

Immediate applications of the work include instrument-less biosensor arrays and living light sources for a range of applications.

The project involves a distinguished international advisory board representing marine biology, molecular biology and biotechnology, materials science, electrical and optical engineering, bioengineering, surface and interface chemistry, physics, and ecology.

The project includes a strong education component involving the Center for Integrated Science Education (CISE), high school teachers, and a local science education products company. Corporate collaboration is strong. Collaborations and technology transfer relationships are already in place. The scientific output from this project will provide significant benefits to the private sector, well within the first year of the project.

3. **THE INSTITUTION:** Signature and typed name and address of university official authorized to obligate contractually, and with whom business negotiations should be conducted.

Ray M. Nilsson — MAR 0 8 1995
(Signature) (Date)

Manager, Grants & Contracts — Ray — M — Nilsson — (801) 581-3008
(Title) (First Name) (MI) (Last Name) (Phone Number, Including Area Code)

University of Utah
Office of Sponsored Projects
Legal Name of Grantee (University)

1471 Federal Way
Street Address (P.O. Box Numbers Cannot Be Accepted)

Salt Lake City — Utah — 84112
(City) (State) (Zip Code)
PHONE: (801) 581-6903

4. **CERTIFICATIONS BY OTHER UNIVERSITY OFFICIALS:** Use this space for names, titles, and signatures of other officials you wish to approve submission of this proposal (e.g., the Principal Investigator and Department Head, Dean or other officials). A separate sheet may be submitted if additional signatures are required. Recall that all sheets must render the entire proposal no more than 50 pages in length.

Joseph D. Andrade, PI — 3/10/95
Principal Investigator (Typed name plus signature) Date

Robert G. Glass, Dir. Office of Sponsored Projects — Date
Other Official (Typed name plus signature)

Other Official (Typed name plus signature) — Date

Objectives/Specific Aims (See Table 1, Time Plan/Schedule):

Specific Aims are divided into three categories: Photoproteins and Ordered Arrays, Artificial Photo-Organs, and Applications-Bio-Photon Based Devices. Table 1 lists each specific aim/objective and various tasks as a function of the three years of the project and the additional two optional years. Figure 1 presents the subjects and questions addressed and the bio-photon devices we expect to develop. Figure 2 shows the general organization and management and includes the groups involved, their expertise, and their general areas of focus and responsibility.

Part I -- Photoproteins and Ordered Arrays:

Firefly luciferase: expression in *E. coli*, purification, characterization, adsorption, mobilization, orientation and ordering at interfaces, stability in the immobilized state, bioluminescent activity, characterization of ordered array bioluminescence with respect to directionality, intensity, and polarization. Engineered firefly luciferase: to enhance its orientation, immobilization, and ordering in arrays. Firefly luciferin and luciferin derivatives: sources, stability, and application.

Bacteria luciferase: culture of marine bacteria under a variety of culture conditions, bioluminescence expression, extraction, purification, and characterization of bacterial luciferase. Expression in *E. coli*, purification, characterization, adsorption in mobilization, orientation and ordering at interfaces, stability in the immobilized state, bioluminescent activity, characterization of ordered array bioluminescence with respect to directionality, intensity, and polarization. Expression of bacterial luciferases in *E. coli* to enhance purification, orientation, and ordering.

Dinoflagellate luciferases: culture of three different species of dinoflagellate under a variety of culture conditions, the study of bioluminescence intensities and characteristics, preliminary study of scintillon morphology and characteristics, extraction, purification, characterization and application of luciferase and luciferin derived from these organisms. Expression in *E. coli*, purification, characterization, adsorption, mobilization, orientation and ordering at interfaces, stability in the immobilized state, bioluminescent activity, characterization of ordered array bioluminescence with respect to directionality, intensity, and polarization.

Expression of engineered dinoflagellate luciferase to enhance orientation, immobilization, and ordering.

Extraction, purification, and characterization of Luciferin-Binding Protein (LBP); expression in *E. coli*.

Part II -- Artificial Photoorgans:

Photoorgans: Careful evaluation and analysis of the properties and nature of bacteria photoorgans in marine species. Analysis of bioprocess engineering aspects of bacterial photoorgans. Development of means to produce high bacterial density cultures, including means to orient and even "order" the bacteria.

Table 1. Time Plan
Years

Task	1	2	3	4	5
General:					
Advisory Board	→	→	→	→	→
Bio-Photon Conf.	→	→	→	→	→
Photoproteins:					
Firefly	→				
E-coli engineered Bacteria	→	→			
E-coli engineered Dinoflagellate	→	→	→	→	→
E-coli engineered	→	→	→	→	→
Photoorgans:					
Photoorgan Bacteria	→	→	→		
Scintillon Dinoflagellates	→	→	→	→	→
Artificial Symbiosis			→	→	→
Application:					
Photo-protein Biosensors					
ATP		→			
$FMNH_2$			→		
Other					→
Cell Biosensors:					
Bacteria		→	→	→	
Dinoflagellates			→	→	→
Living Light Sources:					
Bacteria			→	→	→
Dinoflagellates			→	→	
Photosystems			→	→	→

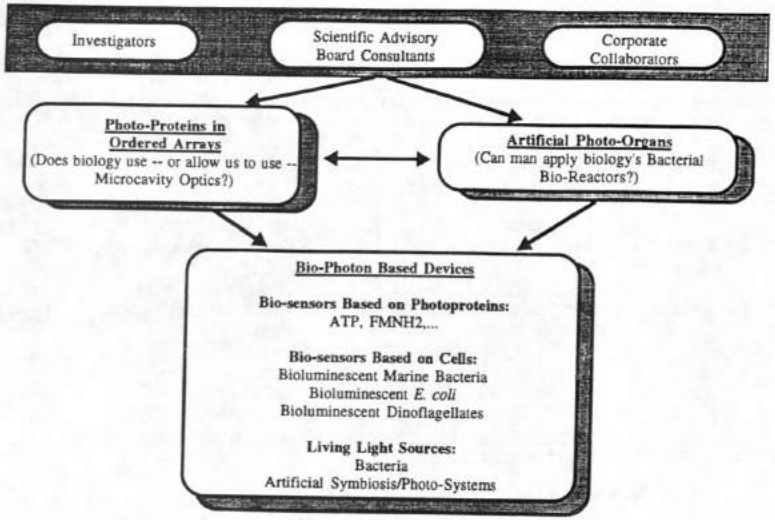
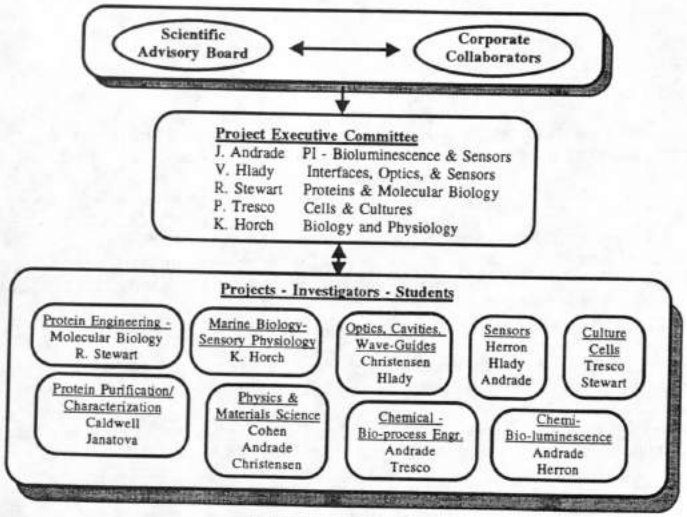


Figure 1: Bio-Photon Project -- Topics, Questions, and Applications.



The Bio-Photon Project:

Figure 2: Organization & Management

Bacteria: High density culture of marine bioluminescent bacteria. Development of means to pack and order marine bacteria in micro-bioreactors. Modification of culture medium and culture conditions to enhance ordering, orientation, and density of the bacteria. Modification of culture media and conditions to enhance total bioluminescent output. Optimization of all conditions to enhance stability and survivability of the artificial photoorgans.

Scintillons: Consideration of structure and function of dinoflagellate scintillons with respect to ordering, packaging, and self-assembly of artificial scintillons.

Artificial Symbiosis: Consideration of co-cultures of photosynthetic bacteria and bioluminescent bacteria, diatoms (photosynthetic) and bioluminescent bacteria, and of dinoflagellates (photosynthetic) and bioluminescent bacteria.

Applications -- Bio-Photon Based Devices:

Photoprotein-based biosensors: ATP based, and $FMNH_2$ based biosensors using optimized ordered photoprotein arrays.

Cell-based biosensors: Use of high density bacteria and dinoflagellate cultures as means to detect toxins and other chemical via bioluminescence inhibition or bioluminescence enhancement.

Living light sources. Use of high density artificial photoorgans as regenerable, lon-lived light sources.

Background and Rationale:

Photons are basic to biology. Biology has learned to efficiently collect photons and transduce and utilize such energy via photosynthesis. Biology also utilizes photons for sensing and imaging purposes via vision. What is not as well known, and indeed has been largely ignored, particularly by the physics and engineering communities, is the fact that biology knows how to produce photons via bioluminescence.

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Although bio- and chemiluminescence are well known processes and are applied in a number of areas, they are generally considered to be impractical as photon sources, in part because the light produced tends to be non-oriented, non-polarized, of relatively low intensity, and to otherwise not have the more precise and well controlled characteristics of sources which utilize modern electro-optical materials.

The growing awareness and application of materials and devices with nano dimensions, and the importance of quantum phenomena in such devices, has led to the consideration of such quantum effects in bioluminescent systems. Although we are unaware of any direct evidence for quantum micro-cavity optics luminescence effects in biology, it may be because bioluminescence processes have rarely, if ever, been approached from the perspective of quantum optics. Proteins and enzymes are colloidal particles of nano dimensions and may be capable of functioning as quantum dots, quantum whiskers, or quantum films. Proteins, and enzymes in particular, often have unique dielectric environments and geometries which can, at least in principle, influence the photon emission process, including both direction and polarization.

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A new Bio-Based Engineering program in our Department of Bioengineering now provides the infrastructure and resources with which to tackle a project on bio-derived optical materials, hence this proposal.

We now have on board a number of new faculty with skills vital to certain components of the project. These new faculty, together with the existing core of faculty in bioengineering, physics, materials science, biochemistry, and pharmaceutics, and our unique Center for Biopolymers at Interfaces, as well as a new Center for Novel Applications of Fibers, provides an infrastructure and capability which permit the Bio-Photon Project to be effectively carried out.

This proposal is at least partially responsive to four different topics in the multi-disciplinary research program of the MURI.

1) Functionally Tailored Textiled Fabrics: [confidential]

Protein Solutions, Inc. (PSI), one of the industrial collaborators, is developing direct reading biosensors utilizing trehalose-stabilized luciferases in the dry state. Synthetic polymer fibers are being used as supports and being impregnated with agarose sols containing stabilized photoproteins. Although this study is very early, it lends the possibility of developing fibers and eventually fabrics which can function as large area, chemically specific biosensors.

Matech, Inc. and PSI both have interests in immobilizing proteins and cells in inorganic and organic matrices, respectively. In PSI's case, they have already demonstrated a long lived culture of dinoflagellates up to 3 years in a totally self contained miniature ecosystem. It is not too far-fetched to expect that one may even be able to maintain such organisms within the confines of hollow fibers, providing literally living, functional fibers and fabrics for unique sensing and other applications. We are studying such systems as potential mammalian cell hybrid organs -- what is commonly called tissue engineering.

These fiber concepts are being considered in the University's new Center for Novel Applications of Fibers. The Center will not only include applications of common synthetic and biofibers, but will also include applications of carbon fibers and human hair, in both cases for sensing and diagnostic applications. Several participants in this project are also involved in the new Center as well as in the Center for Biopolymers at Interfaces.

2) Advanced Biosensor Arrays:

Many of the investigators in this project have worked together for nearly a decade in advanced fluorescence-based wave guide immunosensors. This project, originally funded by ONR some ten plus year ago, has resulted in a large and diverse fluoro-immunosensor effort, which is a major part of the ongoing research program of Dr.s Herron, Christensen, Hlady, and Andrade.

A program on the regulation of antigen/antibody binding for reversible immunosensor applications was funded by the Army Research Office. These two DOD contracts provided the basis for the optical immunosensor program at the University of Utah and provided the resources for the productive collaboration between Andrade, Hlady, Herron, Christensen. Very early stages of the immunosensor concept were substantively focused and enhanced via J. Andrade's interactions and discussions with Ira Skurnick at ARPA (then DARPA).

Both Christensen and Hlady are involved in micromachining and self assembly as applied to chemical bio-sensors. In Dr. Hlady's case, he is making extensive use of photolithographic techniques and atomic force microscopy. Dr. Christensen, in addition to his optical wave guide immunosensor interests and activities, is now involved in projects, on modeling and theoretical studies of optical microcavity-based systems.

Though most of our work to date has involved biosensors for detection of antigens and haptens in liquid samples, our recent experience with the successful preservation of protein structures in the dehydrated state using trehalose and other additives makes it likely that many of these techniques will also be applicable to the monitoring of chemicals in the

vapor and gaseous state. In addition, two new projects on the application of marine bacteria and dinoflagellates to the sensing of toxic volatiles further document the possibilities of our analysis..

3) Advanced Optical Materials (ONR):

Ordered arrays of photoproteins, functioning as optical microcavities, possibly producing oriented and even polarized light, are certainly novel and advanced optical materials. The fact that it may be possible to self-assemble lenses, reflectors, filters, wavelength shifters, and indeed photon production devices from natural materials is intriguing and potentially of wide applicability. These are at least novel and, we hope, "advanced," optical materials and devices.

4) Materials and Processing at the Nano-meter Scale (AFOSR):

Proteins are nano-meter scale machines produced by natural means and self assembled into structures and arrays with unique properties and potential applications, including the possible self assembly of optical microstructures and microdevices.

Specific engineering of the photoproteins involved to facilitate their immobilization, ordering, and self assembly, should make it possible to develop structures and properties which biology has not yet evolved.

Clearly this proposal could be considered non-responsive. We wish to suggest however, that it is at least partially responsive to the above four MURI topics. Indeed we would hope that it might be possible to fund all or a part of the proposal via a multi-agency, multi-topic mechanism. We would look forward to brainstorming and working with various agency and program managers and staff in the development of these novel studies and devices.

Experimental Plans and Methods:

This section is organized in parallel with the Objectives/Specific Aims and Time Plan. Please refer to Table 1. As we discuss each of the tasks and specific aims, please refer to Figure 2, which shows each of the investigators involved and their particular interests and areas of expertise.

General. Scientific Advisory Board and Bio-Photon Conference

We have assembled a Scientific Advisory Board (Table 2) with the background, expertise, and perspective to provide considerable information and insight, as well as with the independence and strength of character to be objective and critical about the project. Funds are budgeted to permit their visit to the project site in Salt Lake City at least once per year. The plan is not to bring them all at once, but rather in groups of two or three for sufficient time to arrange interactions with all relevant parts of the project. We expect that they will be closely involved in those projects/tasks in which they have particular interests, including serving on graduate student thesis committees, and possibly in collaborative research.

Table 2. Scientific Advisory Board/Consultants

Name:	Affiliation:	Expertise:
Baldwin, T.	Texas A & M University, College Station, Texas	A pioneer in the expression of luciferases in *E. coli*, and an expert on the structure and mechanisms of bacterial luciferase.
Kricka, L.	Univ. of Pennsylvania	Perhaps the leading authority on the application of bio- and chemi-luminescence to diagnostic and clinical chemistry and clinical pathology. He also has extensive experience with micromachined analytical devices.
Campbell, A.K.	Univ. of Wales, United Kingdom	Author of *Chemi-luminescence*, and one of the major appliers of bioluminescence in biochemistry and medicine.
Case, J.	UCSanta Barbara, California	Marine biologist with expertise in marine bioluminescence, particularly the distribution and intensities of organisms and the neurosensory/neurophysiological control of bioluminescence.
Herring, P.	Inst. Ocean. Sci, United Kingdom	A leading marine bioluminescence expert and editor of major volumes on the subject.
Hastings, J.W.	Harvard University	One of the world's most active and prolific bioluminescence researchers in whose lab many of the other leading researchers have been trained. An expert on bacterial and dinoflagellate bioluminescence, including the scintillon mechanism in dinoflagellates. Recently expressed dinoflagellate luciferase in *Ecoli*.
Morin, J.	UCLA, California	An expert on fish bacterial photoorgans, as well as on other bioluminescent organisms, including the Cypridina/Vargula group.
Nealson, K.	University of Wisconsin, Milwaukee, WI	A leading researcher in bacterial luminescence, including recognition of the autoinduction mechanism of bioluminescence in some species.

We also expect to have an annual Bio-Photon Conference, not necessarily as a separate conference, but probably tied to a major national or international meeting in which the majority of the Advisory Board members, as well as project staff, would be able to attend. A likely meeting is the International Symposium on Bioluminescence and Chemiluminescence, held every two years. Our own Bio-Photon Conference Meeting would be organized immediately at the conclusion of the major meeting and allow an opportunity for a majority of the participants and advisors to participate in a synergistic group environment.

Photoproteins. Firefly.

Dr. Stewart and co-workers will be responsible for protein expression in *E. coli* and for protein engineering. Dr. Janatova and co-workers will be responsible for protein purification, Dr.s Hlady and Andrade for the interfacial studies, Dr.s Herron and Hlady for the computer modeling, and Dr.s Hlady, Christensen, and Herron for the fluorescence/luminescence characterization.

Since the isolation of the luciferase gene from a number of species, luciferase has become an important tool in the fields of molecular and cellular biology. Recombinant luciferase has been particularly useful as a convenient reporter of gene expression in studies of gene regulation and development in both plants and animals. Much less effort has been directed at using the power of molecule genetics to engineer new properties into luciferase proteins to increase their utility for in vitro applications. Our goal is to design novel luciferase proteins through genetic engineering that will be simple and inexpensive to purify in large quantities, and that can be immobilized in well-defined orientations and densities.

Our initial approach was to add six amino terminal histidine residues to the luciferase gene from the firefly Photinus pyralis (Promega Corp.). The histidine residues bind strongly (Kd > 10-9 M) to immobilized Ni++, allowing for single-step affinity purification of the tagged protein on a metal chelate column. Construction of a histidine-tagged luciferase has been completed and we have shown that the tagged luciferase is expressed at high levels in E. coli and retains bioluminescent activity. We are now in the process of fully characterizing the recombinant luciferase.

The second stage of our research plan is to construct a biotinated luciferase fusion protein that can be immobilized at high densities with a specific orientation to the surface. We will add a segment of the gene encoding E. coli Biotin Carboxyl Carrier Protein (BCCP) to our histidine-tagged luciferase gene. BCCP is involved in fatty acid synthesis in E. coli. Biotin ligase enzyme covalently attaches a biotin molecule to a specific lysine residue of BCCP. Fusion proteins containing the biotin ligase recognition domain of BCCP will contain a single biotin molecule at a specific site as a result of being expressed in E. coli. Because the biotin will be in a specific location, it may be possible for the BCCP-luciferase protein to be immobilized with a defined orientation on a streptavidin or avidin surface. In another approach to building arrays of oriented luciferase we will fuse luciferase to proteins that spontaneously assemble into two-dimensional arrays at lipid water interfaces, or we will add domains to luciferase that specifically recognize a two-dimensiona protein or polymer array.

Once we are capable of producing large quantities of luciferase that can be precisely immobilized we will continue to improve the utility of luciferase through genetic engineering. We may use random mutagenesis to select luciferase mutants that have greater thermal stability, or different photonic properties, e.g., shifted wavelength or narrower bandwidth. It may also be useful to couple the light producing activity of luciferase with other enzyme activities as an approach to regulating luciferase activity in sensor applications.

Existing studies with firefly luciferase will be augmented and expanded. Mr. C-Y Wang is completing a Ph.D. in Bioengineering on Firefly Luciferase, including its incorporation in gels, air drying and reconstitution, and its interaction with lipid membranes. Mr. Wang will stay on as a post-doc at the conclusion of his Ph.D. defense in July, 1995. "Dr." C-Y Wang will oversee and perform much of the day-to-day work on photoproteins. His post-doc studies will be focused on the orientation and 2-Dimensional ordering of firefly luciferase at solid/liquid interfaces. Ordering will also be studied at water/air and water/lipid interfaces, the latter using the techniques developed by Grainger, Ringsdorf, et al. Using fluorescence and bioluminescence microscopy, assembling and

ordering at various interfaces will be studied, including the role of ATP and luciferin in the ordering process. The solid/liquid interface studies by Dr. Hlady and his team will utilize gradient surfaces, total internal reflection fluorescence and luminescence spectroscopy (see Hlady vita).

Mr. Wang will be assisted by several graduate students and will be co-supervised by Andrade, Hlady, and Herron. Herron has utilized computer molecular graphics and protein structure prediction mechanisms to study a variety of protein systems. These computer molecular graphics structural prediction techniques will be applied to firefly luciferase, a protein whose 3-dimensional structure is not yet available.

Wang's studies will include careful consideration of the conformational sensitivity of luciferase, its pH dependence, the role of ATP and luciferin in its conformational stability, and the role of specific ions and ionic strength. The goal is to develop a set of solution conditions and interfacial properties which permit the preparation of monolayers and multilayers of firefly luciferase with controlled orientation and ordered 2-dimensional structure. The films prepared will be characterized with respect to enzyme activity, bioluminescence output, and short-term stability. Such films should permit part of the mechanism of firefly bioluminescence to be established. That is, does the activated luciferin product remain bound during the photo-emission event, or does photoemission occur when the activated oxy-luciferin has been released from the luciferase binding site, a somewhat controversial mechanism proposed by Ugarova and co-workers.

By looking at polarization and de-polarization ratios, both in the fluorescence and luminescence modes, and fluorescence lifetime measurements, the question should be answered.

We will also examine the angular, spatial, dependence of the luminescence, particularly if there is evidence that the activated oxy-luciferin is protein bound during emission. It is possible that the characteristics of the enzyme, coupled with the dielectic nature of, the substrate to which it is immobilized, could provide micro-cavity optical effects which could influence the directionality and possibly the spectral nature of the luminescence.

These studies will utilize native firefly luciferase, as well as that expressed in *E. coli*, using the methods developed by Wood, now in use in our laboratory. Stewart and his group will also produce engineered firefly luciferase modified so as to more efficiently purify and immobiliz e/order the protein at interface.

Bacteria.

Bacterial luciferase will be extracted from large scale cultures of marine bacteria, purified, and characterized by standard methods. This will augment and expand the work already under way in our laboratories.

Bacterial luciferase is a more complex protein in that it involves alpha and beta sub-units and a more complex folding/unfolding behavior.

Studies nearly identical to those described above for the firefly protein will be performed with the intact bacterial luciferase, but with particular attention given to the alpha-beta nature of the molecule and its asymmetric structure. This protein has been more extensively computer modeled, and approximate structures are available, although a full X-ray crystal structure is not yet available. Our expression experiments will also involve means for modifying the bacterial luciferase with appropriate groups and tethers to facilitate oriented immobilization and 2-dimensional ordering, similar to that described above for the firefly protein.

In the bacterial luciferase case it is generally accepted that emission occurs from an immobilized activated state, so here there is less uncertainty about the opportunity to observe directionality of photo-emission and possibly polarization. A set of similar studies will be done as described above for the firefly. The role of the $FMNH_2$ and long chain aldehyde in the interfacial properties of the protein will be considered.

Dinoflagellate luciferase and its bioluminescence mechanism are relatively well known, although there have been less total work than in the case of the bacterial and firefly systems. Dinoflagellate luciferase is a large protein and bioluminescence generally involves a luciferin binding protein, whose behavior is highly pH sensitive.

We will purify dinoflagellate luciferase from several different species of dinoflagellate, including *Pyrocystis lunula*, with which we have the most experience, possibly *Pyrocystis noctiluca* and/or *Pyrocystis fusiformis* and *Gonyaulux polyedra* -- the latter because it is perhaps the most extensively characterized dinoflagellate. In addition to common cell culture extraction and purification, the enzyme will also be expressed in *E. coli* using the recently published methods of Hastings, et al. We also expect to express modified versions of the protein using methods and procedures similar to that discussed above for firefly and bacteria.

By the end of Year 3 we expect to have the 3 major luciferases (bacteria, firefly, dinoflagellate) in large quantities and fully characterized with respect to their interfacial characteristics, including their ability to orient and order at a wide range of interfaces. We expect to have answered the question if there is any directionality and/or polarization, as well as spectral changes, associated with their behavior at a range of interfaces.

The potential behavior of these systems as micro-optical cavities will be modeled by Christensen, et al. using their finite difference time domain (FDTD) technique. We will also model other environments and attempt to predict characteristics of microcavities which might indeed lead to microcavity controlled bioluminescence.

In Years 4-5 we would nano-fabricate such microcavities and immobilize and otherwise incorporate the appropriate luciferases in these environments.

If our Year 1-3 data on oriented ordered layers of photoproteins suggests that there is no particular enhancement of emission directionality, or of other characteristics suggestive of microcavity effects, then we will attempt to induce such enhancement by creating artificial optical microcavities using methods similar to those employed by Yokohama and others. Using the microfabrication expertise in our own Microelectronics Laboratory. Via Dr. Christensen and the expertise of Dr.s Cohen and Hlady, we will

immobilize, orient, and otherwise package these photoproteins in such microcavities, together with means of delivering the appropriate substrates and reagents required for their bioluminescence.

In Years 4-5 we would also add to the Scientific Advisory Board individuals expert in other components of animal photoorgans, and in particular, reflectors. Biology has managed to evolve a range of highly effective structures for the optimal construction and fine tuning of photoorgans. Keeping with our bio-based self assembly approach to luminescent device development, part of our Year 4-5 effort in this area will focus on these biological models.

Photoorgans.

There has been little concerted effort from an engineering perspective to try to design a culture for maximal light emission *in vitro*. This has been done successfully *in vivo*, however, through the symbiosis between bioluminescent bacteria and various bioluminescent fishes, including Flashlight and Pony fishes (Figure 3). Many bioluminescent fishes contain a highly dense and very bright culture of a symbiotic bioluminescent bacteria in their light organ. These light organs have been evolutionarily engineered to produce bright emission with even directional characteristics.

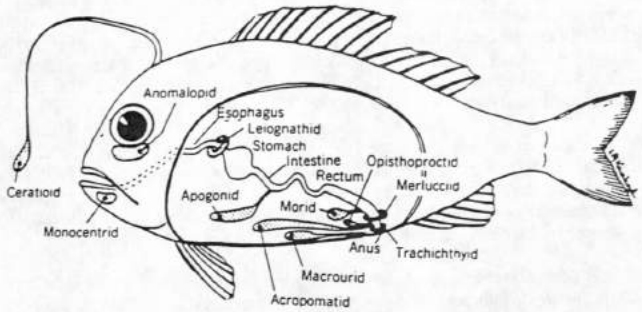

Figure 3. A complex diagrammatic fish indicating the different locations and openings of light organs in the fishes that harbor symbiotic luminous bacteria (from Hastings and Morin).

Working with our marine biology/marine bacteria consultants and advisors and with their colleagues and co-workers, we will develop as complete an understanding as the current literature allows, on the nature and structure of unusually bright bacterial photoorgans. This information will be used to design a set of culture media, bio-reactors, and artificial photoorgan experiments which will facilitate establishment of sets of conditions which we expect will permit selected strains of marine bacteria to be maintained at densities comparable to those found in fish photoorgans. We also hope and expect that

we will be able to control and modulate the culture environment to facilitate the long term viability of such ultra dense cultures, including the enhancement of metabolic pathways which increase the bioluminescence output.

A very interesting feature of some marine bioluminescent bacteria is the phenomenon of auto induction, that is, these organisms do not bioluminesce until they produce a critical concentration of a chemical which then *induces* bioluminescence. The bacteria may be perfectly healthy and non bioluminescent at low concentration; greater numbers are required before the auto inducer reaches sufficient concentration to induce the culture to bioluminesce via a gene activation process. The growth and luminescence of these bacteria is also sensitive to other components in the medium, including salt concentration, iron levels , and over all osmolarity.

Fish light organs house the bacteria in specialized, highly structured, gland-like environments. The fishes have significant anatomical and physiological adaptations for maintaining the symbiotic bacteria and maximizing the luminescence while minimizing the growth of the bacteria.

The bacteria are present at high concentration and have little need to grow or multiply -- they are already present at high and potentially maximum density in light organs. Thus, unlike the growth of bacterial in *in vitro* culture, conditions are such that growth is minimized while luminescence is maximized. The growth rate of bacteria in a fish light organ is of the order of 20-30 times slower than in typical culture conditions. The fish may regulate growth through the regulation of osmolarity. Low osmolarity apparently increases luminescence and luciferase concentration but restricts growth. High osmolarity, comparable to that of sea water, stimulates growth but limits luminescence . This mechanism, although not fully proven, will be important in the design and engineering of an *in vitro* light organ with optimum characteristics.

The bacteria and/or the surfaces and micro architecture of the light organ may be optimized for bacterial immobilization and possibly even orientation. Vibrio harveyi is known to adhere to artificial fiber surfaces under a variety of conditions, including laboratory filters. These observations and these clues will likewise be used in this study to optimize our artificial light organs.

We will conduct a set of studies in developing bacterial culture media in a transparent gel environment. Normal media are constructed in such a way that they are often translucent rather than transparent. We have had extensive experience with the use of agarose and with the ability to control the gelation of agarose via a combination of temperature and agarose molecular weight and other molecular characteristics. Low gelling temperature agaroses have been used by our group for some time to produce luminescent films for teaching and analytical applications.

Given what we learned, and particularly what is known from a reverse engineering study of photo organs, with respect to the chemical and environmental control of the bioluminescent bacterial population, we will begin to modulate and modify our culture material and indeed the design of the devices to facilitate the long term stability of these bacterial cultures, coupled with very high bioluminescence intensity. Control and regulation of osmolarity and pH are the two immediate variables, as well as iron

concentration . We fully expect that in order to develop optimum devices we may need to design a gradient in one or more of these parameters into the device itself.

Much of the culture optimization work will utilize gradient techniques, but modified and enhanced by the gel and solid surface gradient experience in the Andrade and Hlady research groups. We also expect to utilize semi-permeable membranes, and possibly controlled chemical delivery systems, in order to maintain optimum culture conditions in these artificial photoorgans (see Tresco vita).

We do not expect to address the scientific question of the location and distribution of luciferase and its substrates in bioluminescent bacteria, but rather to consider and utilize current literature. We also do not expect, certainly not in the first several years of the project, to be engineering or modifying bioluminescent bacteria in major ways, but rather to use existing strains which appear amenable to high density culture and to artificial photoorgan development.

The Institute of Biophysics in Krasnoyarsk, Russia, has a collection of some 2,000 strains of bioluminescent bacteria. We are already working with them, trying to identify strains which might be unusually sensitive to nicotine and to other drugs. We will work with them to identify strains which may be particularly suitable for *in vitro* high density culture and thus the development of artificial photoorgans.

Dinoflagellates.

Although there is no evidence that dinoflagellates organize or order as photoorgans or components of photoorgans, the fact that many bioluminescent dinoflagellates are photosynthetic, and at least some are capable of being cultured in closed environments for indefinite periods, leads us to consider the possibility of highly dense dinoflagellate cultures whose luminescence could be appropriately activated on demand. This is highly speculative at this stage of our understanding.

We do not anticipate direct studies of the scintillon structures in dinoflagellates, except as related to our studies on the extraction and purification of dinoflagellate luciferase. The dinoflagellate scintillon also includes a luciferin binding protein (LBP). We may indeed have to use LBP in some of our basic studies of dinoflagellate luciferase at interfaces. Through the efforts of our advisor/consultant, J.W. Hastings, we will stay informed and abreast of the scintillon field, and incorporate such studies and understanding in the design of our experiments.

We will also address, particularly in Years 3, and more so in Years 4-5, the issue of co-cultures and potential symbiotic arrangements between various species of bacteria, various species of dinoflagellates, and indeed even between bacteria and dinoflagellates. The role of marine bacteria in dinoflagellates and the possible symbiotic relationships is not well studied, nor understood. It is too early to say what the nature of such artificial symbiosis studies might entail, but we will keep that goal in mind as we proceed through the first three years of the project.

Applications.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests . 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 .

Several years ago we became interested in 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. Protein Solutions, Inc. is now developing a new science educational product, Resurrection™. We have 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 their cell membranes.

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 .

Mr. Wang has succeeded in stabilizing firefly luciferase, together with its substrate, luciferin, in agarose gels containing trehalose 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.

Indeed, our industrial collaborator, Protein Solutions, Inc., is now working on National Science Foundation STTR grant titled: Direct Reading Quantitative Biosensors for ATP-Dependent Processes -- half of the Phase I effort is in J. Andrade's lab at the University of Utah.

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

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 specific sensors for each of those substrates.

One example of a living luminescence system is now being studied in a preliminary manner in Tresco's laboratory, which has a long-standing interest in the development of bioartificial devices as therapeutic implants and as basic science tools. Bioartificial devices are composites of living cells and synthetic or natural biomaterials,

typically semipermable hydrogels, proteins, thermoplastics and inorganic matrices. The Tresco group in collaboration with other members of the research team proposes to build and test a prototype bioartificial light-emitting device capable of directed low-level light emission produced from living organisms. The proposed construct (see schematic) is based on published reports of the cytoarchitecture of the light-emitting organs of marine vertebrates.

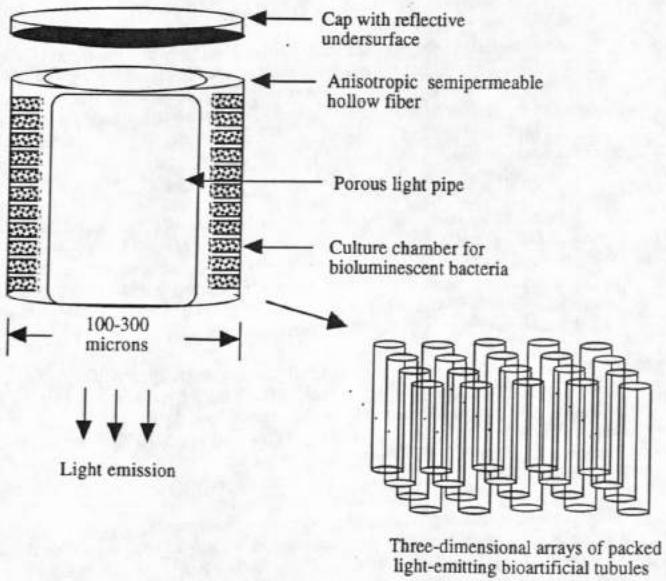

Figure 4. One Possible Living Luminescence Device.

The device consists of arrays of tightly-packed bioartificial-light emitting tubules. Each tubule comprises an anisotropic, semipermeable hollow fiber that possesses a dense perm-selective outer layer and features a lumenal surface that consists of macroscopic cyclindrical pores oriented perpendicular to the long axis of the fiber which serve as culture chambers. Nutrients and bacterial metabolic waste products are transported across the semipermeable fiber wall according to concentration gradients. The highly-folded lumenal interface provides a large surface area for the cultivation of luminescent bacteria. This feature provides tight packing of the bacteria to increase light produced per volume and allows autoinducer substances secreted by the bacteria to up-regulate the expression of luminescent gene products. One end of the device will be sealed with a cap that incorporates a reflective metallized undersurface. A porous cyclindrical silica gel is placed in the lumen of the hollow fiber and will serve as a light pipe. The ends of each tubule are sealed with a transparent photo-cured methacrylate resin. Tubules are packed in a housing

equipped with inflow and out flow ports and a pumping device that circulates microsolutes necessary for bacterial growth around the bacteria-containing semipermeable tubules.

Coupling the tubule system to "gray" water sources may provide a means to remediate human waste while providing a nutrient source for the bacteria, ensuring a long-term low-level light source. Light emitted from bioluminescent bacteria will illuminate the cylindrical silica gel which will be reflected from the metallized cap and directed out the opposite side of the tubule. This device will provide in vitro model system for studying the effect of soluble factors on bioluminescent bacterial cell viability and phenotypic expression, and specifically by examining the potential for bioartificial light-emitting devices.

The building and testing of such a concept will include:

- casting thin films of materials that vary in surface wettability including cellulose acetate, polyacrylonitrile, and polyethersulphone.
- examining luminescent bacterial adhesion to the films to select an optimally adhesive culture interface
- using this material to fabricate semipermeable hollow fibers of appropriate morphology
- establishing bacterial cell loading techniques to seed the lumenal surface of the hollow fibers
- fabricating cyclindrical porous silica gels for incorporation in the lumen of the tubules to serve as light pipes
- assembling the devices and examine light emission as a function of bacterial cell density and device dimensions, including fiber wall thickness, lumen size, and fiber length.

Anticipated Results/Relevance and Significance (see also Background and Rationale):

Ordered arrays of photoproteins will provide films and coatings of enhanced luminescence intensity, much as careful optimization of antibody immobilization has been important in enhancing immunobiosensor performance. There is some probability that properly oriented arrays of luciferases may provide some directional emission due to intrinsic microcavity behavior. This characteristic may be enhanced by co-immobilization and co-assembly with organic structures which provide appropriate dielectric properties.

Such arrays should provide some degree of polarization, although this is less likely for firefly and more likely for bacterial luciferase because of the expected nature of the activated state.

Given what little we know about bacterial bio "reactors" in photoorgans and lacking any evidence to the contrary, we feel that high density, viable marine bioluminescent bacterial cultures are possible. Indeed there is already industrial interest (Protein Solutions, Inc. -- confidential) in developing and marketing such devices as qualitative sensors.

Given our limited experience with dinoflagellates, admittedly a pseudo-eukaryotic cell rather than a prokaryote (bacteria), we feel we can develop long lived dense bacterial cultures (primitive photoorgans), initially using a controlled nutrient delivery system.

Given our limited experience with co-culture of multi-dinoflagellate species -- and also the experience of closed ecosystems and mesocosms, we are confident that an artificial or selective symbiosis is indeed possible (see letter from J. Case).

The real question is how practical and marketable such technologies might be. At least two of our corporate collaborators are betting on this -- and so are we -- although our experience base is too limited at this time to suggest specifics.

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Personnel:

Please also refer to summary vitas, and to Figure 2, Organization and Management.

Joe Andrade, P.I., is Professor of Bioengineering and Materials Science at the University of Utah. He is former Chairman of the Department of Bioengineering and former Dean of the College of Engineering. Joe has worked on proteins at interfaces and biocompatability, biosensors and related areas for 25 plus years, has edited 6 books, and has about 125 peer reviewed technical papers. He is experienced in the management of research organizations and research groups, has produced some 45 Ph.D. and Masters students, consults for a number of industries and government agencies, and has been the founder or co-founder of two companies, including Protein Solutions, Inc., one of the industrial collaborators in this project. He has been working on luciferases and bioluminescence for the last 4 years.

Vladimir Hlady, is a physical chemist/surface chemist/bioengineer who has worked extensively with biosensors, interfacial fluorescence, proteins at interfaces, and colloidal

systems. He is particularly well recognized for his work on protein adsorption and total internal reflection fluorescence as applied to protein adsorption. His present work is focused on scanning force microscopy, protein patterning, and specific protein-ligand binding processes.

Russell Stewart, a new member of the Department of Bioengineering and its Bio-Based Engineering Program, is an expert on proteins involved in motility and transport and has a particular expertise in molecular biology and protein engineering. He is developing micro and nano engineering teaching laboratories together with Dr. Hlady. Dr. Stewart routinely uses protein engineering techniques and *E. coli* to produce proteins with novel engineered properties for his particular research applications. He will be largely responsible for the protein engineering/molecular biology parts of the project.

Ken Horch is a neuro-physiology, sensory systems bioengineer who, earlier in his career, was involved in marine biology, again, from a sensory and neural perspective. Ken is the closest thing we have to a marine biologist and will work closely with the Scientific Advisory Board consultants.

Doug Christensen is an expert on interfacial optics, wave guides, and the development of optically-based biosensors, he is also active in medical imaging and in the applications of ultra sound. Doug has worked in the area of optical microfabrication and in the modeling and analysis of optical systems, including optical microcavities.

James Herron is an antibody crystallographer with extensive experience in protein structure and function, including computer molecular graphics. He now heads up the fluoro-immunosensor activities and is an expert on fluorescence, fluorescence-energy transfer, and the use of proteins as specific recognition elements for biosensors.

Patrick Tresco is also part of the new Bio-Based Engineering Program -- he heads up the tissue engineering laboratories and has a particular interest in the development of devices based on cells in culture, on membranes and in fabric matrices, for potential medical application.

Karin Caldwell is Director of the Center for Biopolymers at Interfaces and an expert in chromatography and separations, as well as in proteins at interfaces. She, together with Jarmilla Janatova, a protein biochemist with extensive experience in protein purification and characterization, will be largely responsible for the protein extraction, purification, and characterization components of the project.

Rick Cohen has worked extensively in the area of luminescence materials and solid state physics and will function as a reality therapist to our thoughts, concepts, and models on the application of microcavity concepts to biochemistry and biology.

On campus advisors include Dr. Richard Normann, Chairman of the Department of Bioengineering and an expert on vision, Dr. Craig P. Taylor, Chairman and Professor of Physics and an expert on luminescence in amorphous systems.

Edward Pope is an expert on sol gel processing and on the immobilization of proteins and cells using sol-gel techniques. Although he is President of Matech, Inc., one

of the industrial collaborators in this program, he also serves as adjunct professor of Materials Science.

Keith Wood with Promega, Inc., another industrial collaborator, is very well known for his work on firefly and click beetle bioluminescence. He is perhaps the most knowledgeable individual on the mechanism of firefly luciferase and was one of the pioneers in the expression of firefly luciferase in *E. coli*.

Facilities:

A number of the investigators in this project are now housed in a new Biopolymers Research Building (Caldwell, Janatova, Stewart, Tresco, Herron, Hlady). The others are housed in the Merrill Engineering Building and the Energy and Minerals Research Center. The laboratories are well-equipped to handle all components of the project. In addition, the Hedco Microelectronics Laboratory, located in the Merrill Engineering Building, provides a wide range of silicon micromachining services.

The Herron and Hlady laboratories are well equipped for fluorescence and luminescence detection using photon counting, as well as other techniques, for sensitive fluorescence and luminescence imaging. Sensitive CCD camera systems and fluorescence lifetime and energy transfer equipment are available. The Herron and Hlady laboratories are well equipped with Silicon Graphics-based work stations and Biosym software for the computer molecular graphics studies.

Christensen's laboratory is well equipped with a variety of fiber optic and wave guide launching, coupling, and characterization equipment, as well as with the computer and related resources for modeling and simulation of optical microcavities and other structures of interest.

The Janatova and Caldwell laboratories are well equipped for protein extraction, purification, and characterization using modern chromatographic and field flow fractionation techniques.

The new Stewart laboratory is well equipped for routine protein engineering and recombinant *E. coli* techniques.

The Tresco laboratory is equipped for a range of cell culture studies.

The Hlady and Andrade laboratories prepare model surfaces for protein immobilization and include techniques for appropriate surface and interface characterization, including X-ray photoelectron spectroscopy.

Equipment Requested:

Although there is a new confocal video microscope, recently obtained as part of the Bio-Based Engineering Program, it is extensively utilized for teaching and existing research activities. Given the major emphasis on cell culture, a full-time advanced microscopy facility is needed in this program.

The Raman and IR equipment is primarily for the cell culture side of the program and for the characterization of culture media and media vapor head space concentrations.

Although CCD cameras are available in the Hlady laboratory, again this program needs almost full time use of photon-sensitive imaging, given the major effort and activities in this project.

We also request a separate HPLC system dedicated to this project. The intense effort in protein modification, preparation, and utilization would greatly overload all existing facilities.

Education and Training:

Graduate students will be derived and selected from the Departments of the various investigators. It is expected that a majority of these students would come from the Bioengineering Program. This program normally receives nearly 1,000 inquiries regarding graduate student admission, receives 150 or so complete applications each year, and normally admits 15-20 students.

A departmental development grant in Bio-Based Engineering, provided by the Whitaker Foundation, a multi-million dollar, multi-year initiative, has permitted the Department to expand its faculty and expand its graduate student program. The students recruited for this project will generally be second year students in the Department of Bioengineering and other participating departments, and will have already completed one year of extensive core course work and research training. They will largely be from the Whitaker Bio-Based track and from the students supported by an NIH training grant in biotechnology and protein engineering.

J. Andrade also co-directs the Center for Integrated Science Education and has close contact with the high school science teacher community. Working through Dr. Brett Moulding, state science curriculum coordinator in the State Office of Education , high school science teachers in the area will be made aware of the summer research opportunities in the Bio-Photon Project. We will develop an application and selection mechanism which will insure that the teachers selected have the interest, motivation, and at least some of the skills to substantively contribute to the work, but we will also insure that those selected will benefit significantly from the experience and will thus be better educated, more highly motivated teachers as they return to their classrooms, i.e., the purpose of this segment of the budget is to both enhance the skills of local science teachers, as well as to provide benefit to the project.

Undergraduate education will be enhanced through the Center for Integrated Science Education (CISE) at the University of Utah, a project of the Colleges of Science, Engineering, and Education, and the University's Undergraduate Research Opportunities Program (UROP). J. Andrade, PI on this project, works closely with CISE and UROP and the project will involve undergraduates through CISE. We will also involve high school teachers as visiting scientists and assistants during the summer months.

The Center for Integrated Science Education is working together with a number of local women's groups, and with the Girl Scouts in programs to encourage women and minorities in science, math, and technology fields. Our staff will be regularly involved in these programs.

Budget & Justification:

Personnel & Salaries. The various investigators and their areas of expertise are summarized in Figure 2, together with their responsibilities and involvement (see section on Objective/Specific Aims).

The individuals budgeted at 0.5 month will not have any major research responsibilities, but will be involved in all meetings, brainstormings, discussions, data analysis and critique of the program. The others will have direct research responsibilities, including graduate student, post-doc, and technician supervision.

The three post-docs will be allocated generally to the photoprotein molecular area, the cell culture and bioluminescence biology area, and the third to applications, optics, and microcavities. It is expected that the eight graduate students, and six undergraduates will be similarly dispersed, although in the early phases of the program there would be greater emphasis on protein modification, preparation, and related topics.

Consultants and their expenses were already discussed in the section on the Scientific Advisory Board. Travel is also requested for the project participants to attend major national meetings and conferences as appropriate.

The equipment items budgeted are not available at the level and commitment which is required for this project. Please refer to separate section on Equipment Requested.

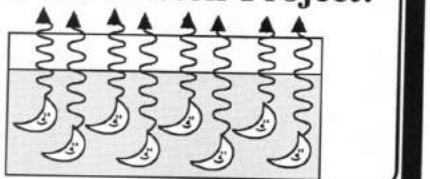
Other costs include standard instrument time charges for characterization and shop services, for microchannel and microdevice fabrication services of the Microelectronics Laboratory, and for surface analysis and characterization.

Technical Support Staff will be largely allocated as follows: 1/2 technician to the molecular biology protein engineering group, 1/2 technician to the protein purification-characterization group, 1/2 technician to the Proteins at Interfaces, Surfaces, and Immobilization tasks, and 1/2 to the cell culture/artificial photo organ area.

The undergraduate students and the summer high school teachers will be allocated in a manner commensurate with their interests and skills, and with project needs.

Although we have not specified an institutional match, the University of Utah has already invested heavily in our Bio-Based Engineering Program -- including a new building, new faculty slots (Stewart, Tresco, Hlady), and start-up equipment. In addition, all graduate students automatically receive a tuition waiver -- another major match.

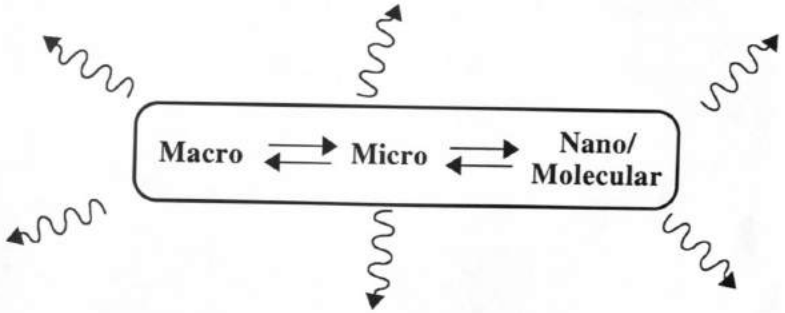
The Bio-Photon Project:



Photoorgans: Performance, Anatomy, Microanatomy, and Molecular Organization

Microorganisms-Bacteria and Dinoflagellates: Enhanced Culture, Microanatomy, Assembly, Induction, and Molecular Organization

Luciferases: Structure, Stability, Mechanism, and Enhancement



A joint project of the

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