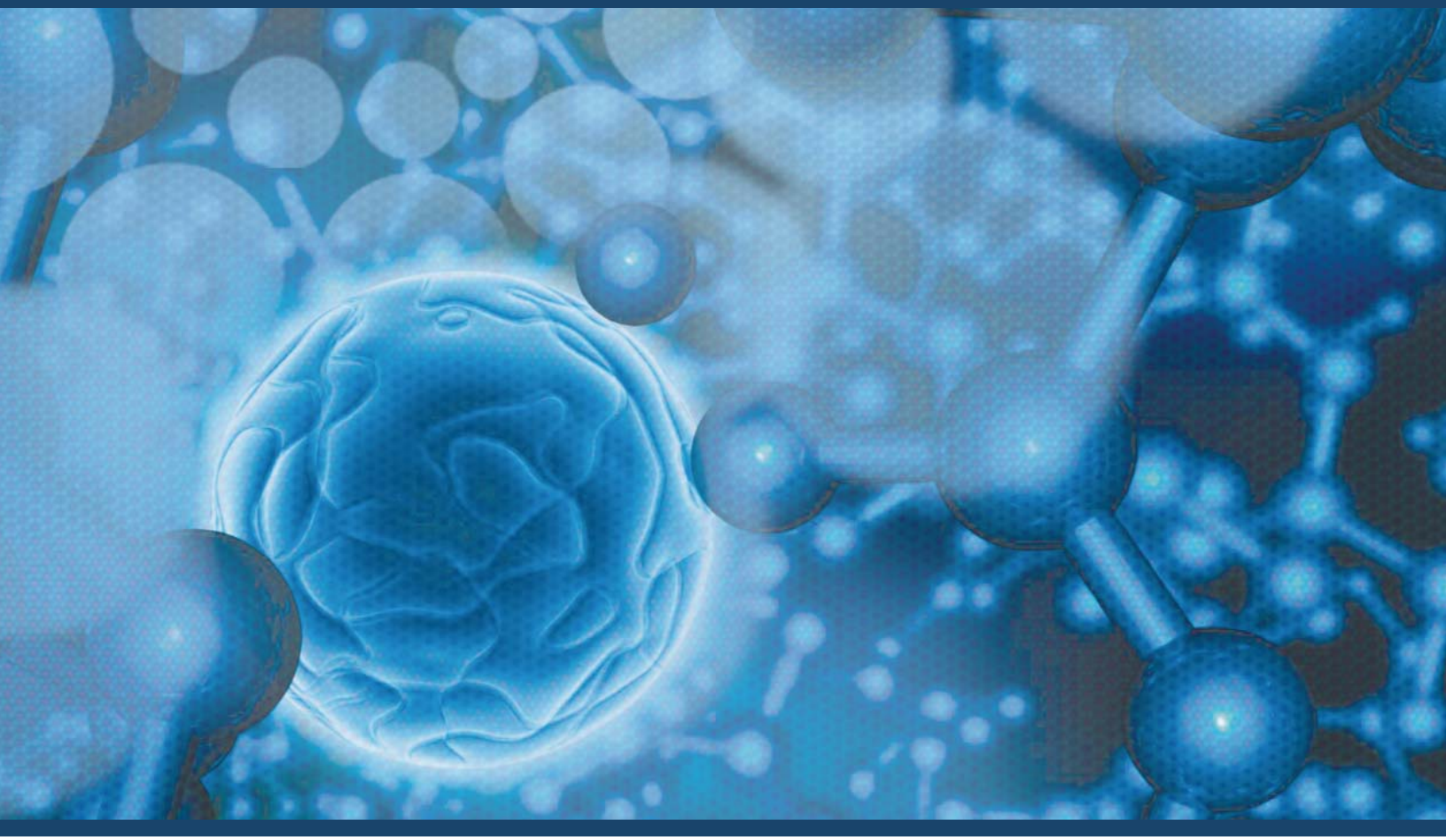
IMAT
Innovative Molecular Analysis Technologies

NCI's Innovative Molecular Analysis Technologies (IMAT) Program: Program Evaluation

2008



IMAT
Innovative Molecular Analysis Technologies

NCI's Innovative Molecular Analysis Technologies (IMAT) Program: Program Evaluation

2008

http://imat.cancer.gov


NATIONAL CANCER INSTITUTE
National Cancer Institute


OTIR
Office of Technology
and Industrial Relations


OBBR
Office of Biorepositories
and Biospecimen Research


Division of
Cancer Biology


DCP
Division of
Cancer Prevention


Division of
Cancer Control and Population Sciences


DIVISION OF CANCER EPIDEMIOLOGY & GENETICS


DCTD
Division of Cancer Treatment
and Diagnosis

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

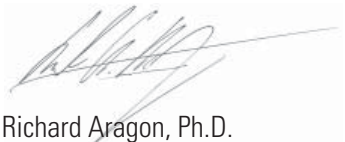
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

In 1998 the National Cancer Institute (NCI) established the groundwork for a highly successful program focused on early-stage technology development to meet the specific needs of cancer researchers and clinicians by stimulating the next wave of technologies capable of being applied toward the field of cancer research. Unlike other initiatives of the time, the Innovative Molecular Analysis Technologies (IMAT) program solicited only the most cutting-edge ideas, thus restricting its application pool to those projects with the potential to be truly transformative. By doing so, the program filled a void that no other program at the NCI or the National Institutes of Health (NIH) filled.

Taking risks on early-stage transformative technologies, IMAT has contributed to many of the blockbuster technologies that are now on the market and in almost ubiquitous use across the cancer research and clinical communities. Successfully commercialized products such as RNALater, Affymetrix gene chips, Illumina bead platforms, and quantum dot labeling technology were all considered high-risk ideas at the time of their inception and initial funding through the IMAT program. Yet, their current widespread use and applicability to multiple clinical and basic sciences research settings are a testament to the high payoff and impact that such transformative technologies have provided to the field of cancer research. By soliciting and supporting these otherwise risky technologies through the IMAT program, the NCI has not only supported the development of these new transformative technologies in and of themselves but also supported them in a manner consistent with providing researchers rapid access to such platforms through appropriate commercialization and dissemination. The NCI has thus taken risks to substantiate the ultimate value and utility of such technologies even in cases where venture capital firms may have been reluctant to do so because of the inherent risks associated with innovative technology development.

Currently, there are new challenges facing cancer researchers and clinicians, and as such, the need for a sustained technology development pipeline encompassing inception and initiation (i.e., the "bright idea" stage) through dissemination and commercialization has never been greater. Challenges represented by the need to rapidly assess all of the epigenetic changes in single cells, directly measure microenvironment impact on cancer metastasis, and collect rare cells from the blood of patients with recurrent disease require *creative thinking* and *risk-taking* to enable research in a manner similar to the way that gene expression profiling is currently enabled. IMAT seeks to fill this void by (1) empowering small commercial entities such as small business concerns to think creatively, (2) stimulating such firms or their technologists to partner with biologists and clinicians who face similar technical challenges, and (3) taking the risk needed to break through common technical barriers that currently impede research and effective clinical decision-making. By accomplishing these goals, the IMAT program seeks to stimulate progress in the field of cancer research at a pace that is *revolutionary* rather than evolutionary and to ensure the adequate and equal dissemination of knowledge that stems from such an approach.

In 2006 the NCI Executive Committee commissioned an external evaluation of the IMAT program in order to assess the program's progress toward meeting its stated objectives. The Office of Technology and Industrial Relations, in conjunction with the Office of Biorepositories and Biospecimen Research, is thus pleased to provide a copy of this evaluation to members of the NCI Executive Committee. This evaluation provides an assessment of the program's progress and lays the groundwork for its continued future success in keeping the forefront of cancer research moving forward.



Richard Aragon, Ph.D.
Program Director, Innovative Molecular Analysis Technology Program
Office of Biorepositories and Biospecimen Research
Office of Technology and Industrial Relations
National Cancer Institute

Contents

NCI's Innovative Molecular Analysis Technologies (IMAT) Program: Program Summary

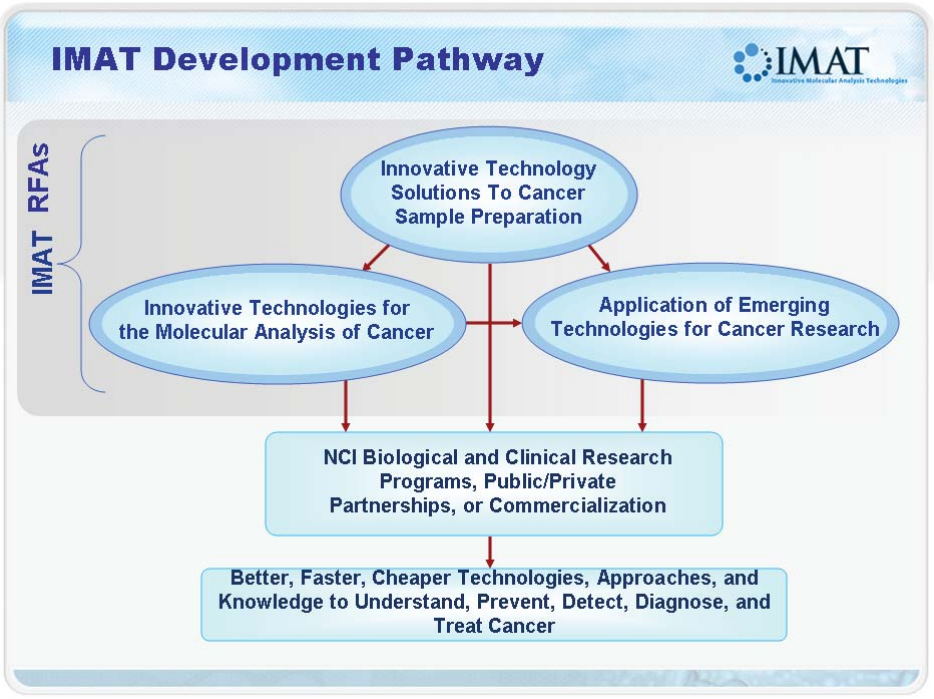
Cancer is the second leading cause of death in the United States (National Center for Health Statistics, http://www.cdc.gov/nchs/fastats/lcod.htm). The National Cancer Institute (NCI) has long been waging a battle against this devastating disease. In recent years the National Institutes of Health (NIH), under its Roadmap initiative, has increased its efforts to accelerate scientific discoveries related to the causes, diagnosis, prevention, and treatment of this disease and to disseminate such knowledge more quickly into clinical and preventive practice as well as into patient care. As part of this strategy, the NCI and the NIH have increased emphasis on the funding of innovative approaches that not only could potentially have a greater impact but also carry a greater amount of corresponding risk. Such approaches may ultimately reduce the incidence and improve the outcomes of cancer. In the shorter term, it is hoped that the strategy will increase knowledge about the etiology, pathophysiology, genetics, and pharmacogenetics of cancer. One critical area of such a strategy is basic research at the molecular level. Analysis of the structures and processes at this level requires sophisticated technologies that have only recently started to become available. Also, the development of advanced computers and software applications has increased the efficiency and effectiveness of discovery. However, it is recognized that even more sophisticated and advanced technology is needed.

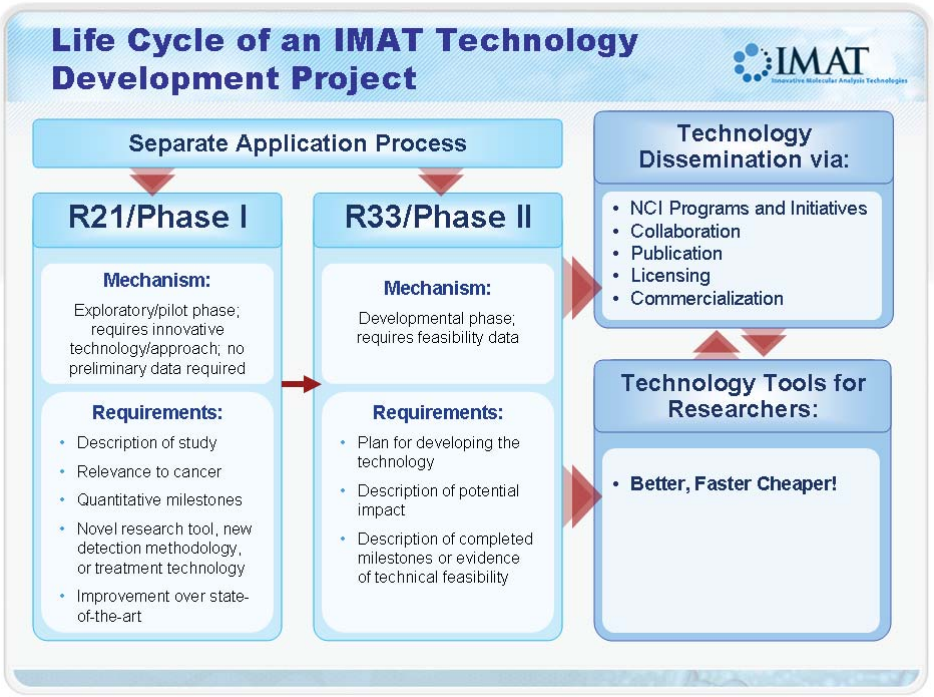
Against this backdrop, the NCI established the Innovative Molecular Analysis Technologies (IMAT) program in 1998 with three objectives:

1. *To focus innovative technology development efforts on the field of cancer,*
2. *To solicit highly innovative technology development projects from the scientific and clinical communities, and*
3. *To accelerate the maturation of meritorious technologies from feasibility to development and/or commercialization.*

The intended purpose of the IMAT program is thus to solicit and fund highly innovative, high-risk, cancer-relevant technology development projects in order to achieve the aforementioned objectives. As a transdivisional, research-enabling program, IMAT has sought to leverage its capabilities in bringing together a multidisciplinary group of scientists, technologists, and engineers to address cancer-relevant problems and to expand technology development interests across the NCI and the NIH. The program utilizes the R21 and R33 mechanisms to allow for peer review of high-risk, high-innovation technology platforms and approaches. IMAT program RFAs solicit R21, R33, and SBIR/STTR applications for three rounds of review each year.

Through solicitation, outreach, education, and integration of the investigator and technology communities, the IMAT program seeks to capitalize on its prior successes in focusing a diverse spectrum of new and emerging technologies on cancer research. The program hopes to continue to stimulate the inception, development, and subsequent application of cancer-related technologies, some of which have gone on to support the acquisition of basic knowledge about multiple types of cancer, thus feeding the R01 and cancer discovery pipelines. The program also hopes to capitalize on its successes in integrating and accelerating meritorious technology development projects by providing funding opportunities and/or a minimal funding gap between the development of new technologies and the demonstration of technological feasibility, particularly for the most highly innovative technology development projects, despite the fact that such projects may also be of higher risk.


IMAT Development Pathway
IMAT
IMAT RFAs
Innovative Technology Solutions To Cancer Sample Preparation
Innovative Technologies for the Molecular Analysis of Cancer
Application of Emerging Technologies for Cancer Research
NCI Biological and Clinical Research Programs, Public/Private Partnerships, or Commercialization
Better, Faster, Cheaper Technologies, Approaches, and Knowledge to Understand, Prevent, Detect, Diagnose, and Treat Cancer


Life Cycle of an IMAT Technology Development Project
IMAT
Separate Application Process
R21/Phase I
Mechanism:
Exploratory/pilot phase; requires innovative technology/approach; no preliminary data required
Requirements:
Description of study
Relevance to cancer
Quantitative milestones
Novel research tool, new detection methodology, or treatment technology
Improvement over state-of-the-art
R33/Phase II
Mechanism:
Developmental phase; requires feasibility data
Requirements:
Plan for developing the technology
Description of potential impact
Description of completed milestones or evidence of technical feasibility
Technology Dissemination via:
NCI Programs and Initiatives
Collaboration
Publication
Licensing
Commercialization
Technology Tools for Researchers:
Better, Faster Cheaper!

Evaluation Purpose and Objectives

Because the IMAT program has not been subjected to a formal evaluation since its inception, the implementation of an effective evaluation construct and strategy to adequately assess the program is both timely and necessary. The purpose of the study described herein is thus twofold: (1) to determine the feasibility of conducting an outcome evaluation of the NCI's IMAT program, given the program's structure and transdivisional nature, and (2) to design such an evaluation in a manner that is comprehensive, timely, and meaningful. To achieve these objectives, the IMAT program pursued and was awarded external funding from the NIH Office of Evaluation and conducted both a feasibility study and subsequent outcome evaluation for the program. Several critical elements would affect both whether an outcome evaluation could be conducted and the nature of that outcome evaluation. These elements included:

- *Outcomes.* Any conceptual framework developed for evaluating the IMAT program should be clear about the outcomes. We know that it is too early to assess long-term outcomes, such as reduction in the incidence of cancers, or perhaps even outcomes related to integration of findings into cancer prevention, diagnosis, and treatment. Shorter term outcomes related to generating and disseminating knowledge, creating scientific collaborations, and pushing clinical studies forward are likely measurable to some degree. The feasibility study should therefore elaborate on the possible outcomes and describe their relationship to the IMAT program through a conceptual framework.

- *Supported Technologies.* Although an evaluation should not be concerned with the technical aspects of the technologies employed, since the technologies themselves are not subject to assessment within a program evaluation framework, it should focus on describing the expected impact of the technologies in terms of how they will be used and the length of time until they are used practicably by practitioners or researchers. In other words, it is important to understand how the technologies will affect any possible outcomes, and in doing so some classification of the technologies should be developed. This information should be gathered from interviews with knowledgeable individuals.

- *IMAT as an Intervention.* In program evaluation terms, the IMAT program is the intervention. That is, the IMAT program provides funding that is expected to result in greater returns than had the funding been used for other initiatives. In an ideal situation, the IMAT program would have provided the same kinds of opportunities to researchers throughout its 8-year existence. However, with the recent modifications to the original IMAT program, that continuity may have been interrupted. In other words, the emphasis on the two new areas may be expected to result in outcomes that are somewhat different from those prior to their introduction. The question therefore is whether projects funded recently can be evaluated in the same context as previous projects. Also important in this context is whether the program emphasis on innovation changed throughout the period. It would be useful to examine the ratings for all responses to the RFAs during this period and compare them across different funding periods.

- *Other NIH Research Opportunities.* The NIH offers a large array of other funding opportunities to help establish centers that allow collaboration in certain kinds of research, assist in obtaining and sometimes modifying biomedical instrumentation, and conduct research. The availability of these other funding opportunities to IMAT investigators may leverage their own efforts. Thus, understanding the interplay between these other opportunities is important in evaluating the IMAT program.

- *Research Environment.* Increasingly, researchers are encouraged to be more collaborative, working across disciplines and institutions. These arrangements are expected to be more effective in bringing intellectual resources to bear on research problems of consequence. Individuals now also have access to a variety of instrumentation, including bioinformatics applications, that were not available a decade ago. The research environment has changed and become more complex. Describing the availability and use of

this environment to IMAT-funded researchers is important to characterize for the purposes of the outcome evaluation.

- *Participants.* Researchers can be funded through R21/R33 mechanisms as well as mechanisms for small businesses (SBIR). The latter are tailored to facilitate commercialization of the technologies developed under the auspices of small businesses. This differentiation, as well as other basic characteristics associated with the research teams, may be important in examining how the technologies were developed, tested, and brought to market. Another important factor relates to information sharing when businesses are focused on maintaining proprietary information.

Evaluation Design

The feasibility study used to inform the IMAT evaluation focused on six broad research questions:

1. *Is it feasible to conduct an outcome evaluation of the IMAT program?* (Overall, is it appropriate to conduct an evaluation of the IMAT program? If so, what is the optimal approach to conducting the evaluation? What is the estimated time under which this evaluation can be completed?)

2. *What evaluation questions are answerable, and, when answered, will they provide meaningful and useful information about the IMAT program?* (Based on the objectives of the IMAT program as stated above, what specific evaluation questions should guide the study? Will these questions provide meaningful data that will allow for the assessment of progress made toward IMAT program goals?)

3. *What measures can be used to answer the evaluation questions?* (What performance measures will reveal whether the IMAT program goals are being achieved?)

4. *What data are needed to answer the evaluation questions?* (Are data currently available? If not, what steps are required to obtain the necessary data?)

5. *What is the most appropriate and cost-effective method for collecting and analyzing the data for the measures?* (What type(s) of research methods can be employed to collect and analyze the IMAT program data? How will these method(s) be implemented? What are the limitations, if any, of these method[s]?)

6. *How much time would be required to collect and analyze the data?* (Given the type[s] of research methods employed and the availability of data, how long will it take to collect and analyze the IMAT evaluation data? Will OMB clearance be required for data collection? If yes, how will this impact the estimated timeframe for the evaluation?)

Answers to the above questions were pursued via an evaluation construct and strategy that incorporated the following elements:

- *Clarification of Issues and Objectives.* A project officer was designated as the lead point of contact for the evaluation and for subsequent interactions between the evaluation contractor/staff and the IMAT program. The contractor met with the Project Officer to discuss the following items and to inform the development of the evaluation:
  - The program to be evaluated and its goals
  - The information sought from the evaluation
  - How the results of the evaluation will be used
  - The timeliness of the evaluation

- *Identification and Review of Relevant Literature.* The designated contractor was asked to conduct a comprehensive review of relevant literature or related studies to determine the feasibility of an evaluation and to inform the evaluation design.

- *Identification of Relevant Stakeholders.* The contractor identified persons directly involved or likely to be involved in or affected by the evaluation so that the evaluation can benefit from their participation and address their needs.

- *Development of Study Questions.* The contractor developed a proposed set of study questions for the evaluation to answer. The questions considered which processes, outcomes, and implementation effects are most useful to learn about. Questions addressed the stated purpose of the evaluation and the achievement of the specific objectives of the evaluation.

- *Identification of Key Variables.* The contractor was asked to identify and describe the target populations(s) of interest and to determine what information is needed to answer each study question, including variables that describe the program resources and activities, population characteristics, and external factors.

- *Review of Existing Data.* The contractor conducted a review of existing data sources to provide information on the key variables in the evaluation design.

- *Development of a Conceptual Framework for the Evaluation.* In consultation with the IMAT program staff, the contractor developed a conceptual framework to illustrate how the IMAT program is intended to work to achieve its goals, including a diagram of the framework.

- *Establishment of a Plan for Data Collection and Analysis.* The contractor determined the types of data that would be used to answer the study questions and identified feasible performance and comparison measures. Strategies were determined to ensure data integrity and to address ethical considerations such as burden on respondents, confidentiality, and security of gathered information.

- *Recommendation for and Pursuit of Evaluation Design.* The contractor recommended and pursued (an) evaluation design(s).

Specifically, a multistage approach was developed for conducting the evaluation. The first stage involved review of grant applications and analysis of data on their overall and subcomponent ratings. A literature review was also performed, with the resulting information used to generate a protocol for conducting interviews with NCI staff members associated with each of the three IMAT areas. Staff members were interviewed about objectives, process, and the projects that have been funded. The primary aim underlying such interviews was to describe the IMAT program as an intervention and to understand why certain funding decisions were made. Stakeholders were also more easily identified through these interviews.

The second stage involved interviewing nine IMAT principal investigators (PIs), three from each program area (i.e., Innovations, Emerging Technologies, and Sample Preparation). These interviews were aimed at understanding how grantees operate in the field, the extent to which they collaborate, their perception of the eventual outcomes of their work, their timeframes for development and next steps, and how such grantees use institutional and other resources in their IMAT-sponsored research.

In answering these questions, the two major challenges faced in terms of developing an outcome evaluation were whether the IMAT program can be characterized as a relatively fixed set of opportunities for researchers and whether the complexity of the environment would confound any results that could affect associating IMAT

opportunities with outcomes. In the first regard, the IMAT program, as discussed above, presents a program in which long-term outcomes could be difficult to evaluate but in which short-term outcomes or outputs may be less difficult. A potentially confounding effect is whether the IMAT program of 8 years ago is the same as the current program and whether the current IMAT format has been in existence long enough to allow for the observation of outcomes. The complexity of the research environment presents two evaluation issues: the degree to which the research environment has changed since the inception of IMAT and the degree to which it can be adequately identified in order to understand IMAT outcomes relative to outcomes associated with other NCI programs or non-NCI initiatives.

Evaluation Results

The NCI's IMAT program supports research projects that are developing creative methods and tools for understanding, preventing, diagnosing, and treating cancer. The IMAT program accomplishes its objectives by supporting and funding highly innovative, high-risk, cancer-relevant technology development projects.

Twelve closely related IMAT Funding Opportunity Announcements (FOAs) are currently open. Each of these FOAs has been assigned to one of the following three related thematic components.

Innovative Technologies for the Molecular Analysis of Cancer

This set of Request for Applications (RFAs) supports the development of novel technologies that can be used for the molecular analysis of cancers and their host environment in support of basic, clinical, and epidemiological research. In particular, these RFAs solicit technologies that can:

- Detect alterations and instabilities of genomic DNA
- Measure the expression of genes and gene products
- Analyze and detect gene and/or cellular products, including posttranslational modification and protein function
- Identify and characterize exogenous infectious agents in cancer
- Assay the function of major signal transduction networks involved in cancer

The Innovative Technologies for the Molecular Analysis of Cancer component of IMAT has approximately $3 million in set-aside funds for R21 and R33 awards and $1 million for SBIR/Small Business Technology Transfer (STTR) awards for FY 2008.

Application of Emerging Technologies for Cancer Research

This component supports projects to evaluate the usefulness of emerging technologies in appropriate biological contexts to assess reproducibility and produce preliminary data in response to a biological or clinical question. In particular, these RFAs solicit technologies that are:

- Entirely novel
- Emerging but not currently in broad-scale use
- Currently in use for one application or set of applications and being evaluated for alternative applications

The Application of Emerging Technologies for Cancer Research (AETCR) program has approximately $3 million in set-aside funds for R21 and R33 awards and $1 million for SBIR/STTR awards for FY 2008.

Table 1. Current IMAT RFAs

Funding Mechanism	Project Type	General Requirements	**Theme 1** Innovative Technologies for Molecular Analysis of Cancer	**Theme 2** Application of Emerging Technologies for Cancer Research	**Theme 3** *Innovations in Cancer Sample Preparation*
R21 (Research Program Grant)	Exploratory-pilot	**Required:** Innovative technology/ approach; quantitative milestone **Not required (but allowed):** Preliminary data	RFA-CA 07-033	RFA-CA 07-035	RFA-CA 07-037
R33 (Research Program Grant)	Developmental	**Required:** Feasibility data	RFA-CA 07-034	RFA-CA 07-036	RFA-CA 07-038
R43/R44 (Small Business Innovation Research [SBIR])	Exploratory/ Phase I or Developmental/Phase II	***Phase 1*** **Required:** Innovative technology/ approach; quantitative milestones; small business-specific requirements **Not required (but allowed):** Preliminary data ***Phase 2*** **Required:** Feasibility data and successful completion of Phase I project; small business-specific requirements (including commercialization plans)	RFA-CA 07-039	RFA-CA 07-041	RFA-CA 07-043
R41/R42 (Small Business Technology Transfer [STTR])	Exploratory/ Phase I or Developmental/Phase II	***Phase 1*** **Required:** Innovative technology/ approach; quantitative milestones; small business-specific requirements **Not required (but allowed):** Preliminary data ***Phase 2*** **Required:** Feasibility data and successful completion of Phase I project; small business-specific requirements (including commercialization plans)	RFA-CA 07-040	RFA-CA 07-042	RFA-CA 07-044

Innovations in Cancer Sample Preparation

This program supports the development of novel sample preparation technologies that are suitable for molecular analyses of cancer cells and their host environments. These RFAs support research projects to:

- Develop and significantly enhance or adapt sample preparation methodologies and technologies
- Develop assays to assess sample quality
- Elucidate the criteria by which to judge sample quality

The Innovations in Cancer Sample Preparation (ICSP) program has approximately $1 million in set-aside funds for R21 and R33 awards and $1.25 million for SBIR/STTR awards for FY 2008.

The current RFAs and their requirements are listed by thematic component in Table 1.

A summary of funding by IMAT's three thematic components is provided in Figure 1.

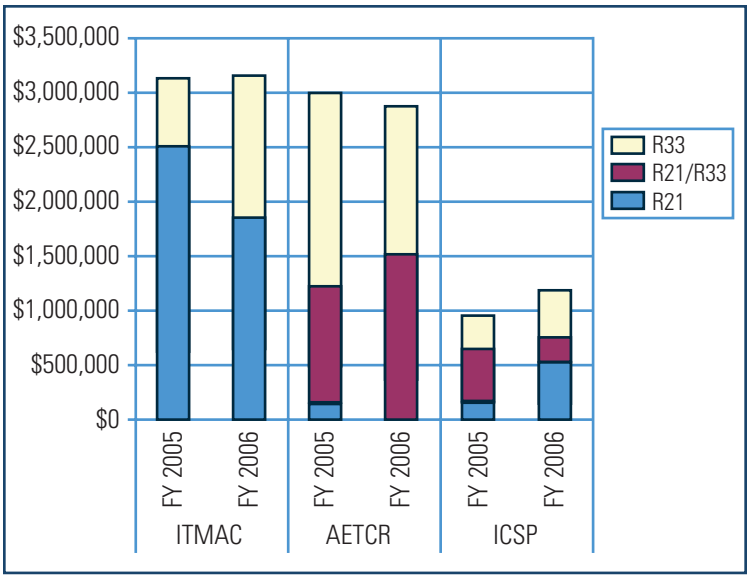


Figure 1. IMAT funding by component and mechanism, FY 2005 and FY 2006.

Specific Program Accomplishments/ Case Studies

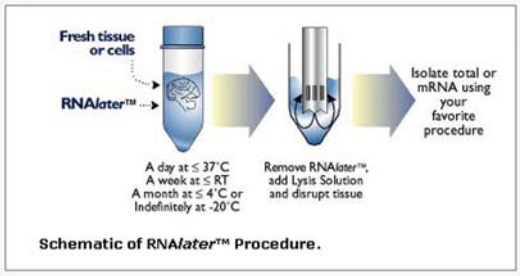
IMAT FOAs support the development of molecular analysis tools that enable the more in-depth examination of the molecular basis of cancer and the identification of individual molecular characteristics that are pertinent to the pathological progression of cancer and its prognosis. Such tools facilitate the identification of genetic factors that influence an individual's risk of developing cancer and his or her ability to respond to adverse external/environmental factors such as radiation, carcinogens, and therapeutic agents.

The IMAT program has successfully promoted cancer-relevant applications of a diverse spectrum of new and emerging technologies. The program has focused on both the inception and development of cancer-related technologies. Some of the technologies originally developed with IMAT funding have been used to facilitate basic cancer research, which feeds the discovery pipeline. Other IMAT-supported technologies have been applied to clinically important questions.

Several case studies are provided in the following table as examples of the development of products in which IMAT played a critical role.

Gary Latham, Ph.D., *Ambion, Inc.*

- **IMAT Award: Enzymatic Tools for Degrading Tissue and Preserving RNA *(2001)***
- **Current SBIR Phase II Awardee (*2005-2007*)**
- **Impact: Flexible handling of tissue samples without significant loss of RNA integrity**
- **Patent Pending (application serial number 60/514,313)**
- **Commercially released by Ambion in February 2005**



Schematic of RNA*later*™ Procedure.

MELT™ Total Nucleic Acid Isolation System (Ambion, Inc.)

IMAT Awards That Contributed to the Development of MELT™					
PI	**Institution**	**Title**	**IMAT Award**	**IMAT Funding**	**Dates**
Gary Latham	Ambion, Inc.	Enzymatic Tools for Degrading Tissue and Preserving RNA	R43CA097482	$162,308	2002-2003
Gary Latham	Ambion, Inc.	Ultra Rapid Methods for Streamlined Tissue to RT-PCR	R44CA097482	$768,803	2005-2007

The MELT™ Total Nucleic Isolation System was developed to isolate RNA with minor protocol modifications. The MELT™ system can process freshly procured or flash-frozen tissues.

The patent-pending MELT™ system allows the hands-free disruption of several solid tissues at the same time without the need for tissue grinding or a polytron, which can create toxic aerosols. MELT™ can be used to lyse samples in a closed-tube format without cross-contamination. The system can liquefy up to 10 mg of fresh or frozen tissue at room temperature in less than 15 minutes using a combination of catabolic enzymes and a small-molecule RNase inhibitor.

After digestion, the RNA or DNA is purified using a streamlined process based on Ambion's MagMAX™ magnetic bead technology (patent pending). The purified RNA or DNA can be eluted from the beads in concentrated form in as little as 15-20 µL. Ambion has validated the MELT™ technology and verified that the purified RNA is functionally equivalent to traditionally isolated RNA.

The IMAT award to Dr. Gary Latham at Ambion, Inc., was first funded in FY 2003 as an IMAT Phase I SBIR, and the funds supported development of the MELT™ system. The research funded by the Phase I award showed that the technology was feasible for further development.

Dr. Latham and colleagues filed a provisional patent application (serial number 60/514,313) for the technologies developed with funding through the IMAT Phase I SBIR award.

Ambion commercially released the MELT™ Total Nucleic Acid Isolation System in February 2005. The Ambion investigators successfully competed for the IMAT R44 Phase II SBIR award, which was funded from 2005 to 2007. The Phase II award is being used to further optimize the reagents for greater stability of RNA, reduce procedure time, and achieve greater RNA purity, including efforts to make the product compatible with Ambion's RNAlater® tissue-stabilizing reagent.

Rudolf Aebersold, Ph.D., *Institute for Systems Biology*

- **IMAT Award: Isotope Coded Affinity Tags (*ICAT*) for Quantitative Proteomics (*2000*)**
- **Impact: Large-scale analysis of complex samples, including whole proteomes and small-scale analysis of sub-proteomes made possible.**
- **PubMed: 218 publications on ICAT technology; 34 with focus on cancer.**
- **7 patent applications filed, one patent granted (Methods for Isolation and Labeling of Sample Molecules, Patent 7,183,116).**
- **Patents Filed:**
  - Methods for Isolating and Labeling Sample Molecules
  - Methods for High Throughput and Quantitative Proteome Analysis
  - Methods for Rapid and Quantitative Proteome Analysis
  - Androgen-Regulated Genes and Uses for Diagnosis, Prognosis,and Treatment of Prostate Neoplastic Conditions

Isotope Coded Affinity Tags (ICAT™) (Institute for Systems Biology)

IMAT Award That Contributed to the Development of ICAT™					
PI	**Institution**	**Title**	**IMAT Award**	**IMAT Funding**	**Dates**
Rudolf Aebersold	Institute for Systems Biology	Isotope Coded Affinity Tags for Quantitative Proteomics	R33CA084698	$1,774,799	2000-2003

Understanding the associations made by proteins within cells that let them perform their different functions is a major goal of proteomics research. In the past, this research was done on one protein at a time. The ICAT technology makes it possible to identify and quantitatively analyze many proteins at the same time in biological samples, such as cell and tissue extracts and biological fluids, using mass spectrometry. This method can be used for both large-scale analysis of complex samples, including whole proteomes, and small-scale analysis of subproteomes.

Dr. Rudolf Aebersold and colleagues first described the initial proof-of-principle test of the ICAT™ technology to quantitate protein mixtures in *Nature Biotechnology* in 1999.[1] This technology has been widely applied, and this seminal publication has been cited 1,576 times to date. Dr. Aebersold further developed the technology through IMAT award R33CA084698. The aim of the IMAT R33 award was to design a new approach for measuring quantitative protein profiles in complex biological mixtures. The project's goals included developing the ICAT™ reagents, liquid chromatography/mass spectrometry (LC/MS) tools, data processing tools, and technology protocols.

The success of the research funded by the IMAT award is evident in the patent applications and commercialization of the technology. Dr. Aebersold and colleagues have filed seven patent applications based on this research.[2] All of these applications cite the IMAT phased innovator award, and one patent has been granted as a result of these applications (Methods for Isolation and Labeling of Sample Molecules, Patent 7,183,116).

Applied Biosystems made the ICAT™ reagent kit commercially available in April 2001 and a second generation of ICAT reagents in 2002. The technology for ICAT™ applications has been incorporated into the informatics tools developed by the major MS instrument manufacturers, and Applied Biosystems has commercialized the Pro ICAT™ software for processing data from ICAT™ LC/MS applications.

Both the Institute for Systems Biology and Applied Biosystems have continued their research on this technology beyond the grant funding period to broaden the detection of proteins, including proteins modified after translation. Additional work has gone into enhancements of the LC/MS tools, techniques, and software.

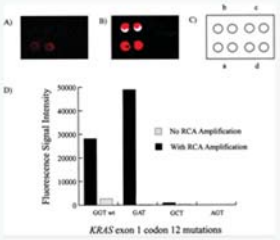
ICAT™ is usually used to find and identify differences in the complex expression patterns of different samples (protein profiling). But ICAT™ has also been used with quantitative MS to find proteins with specific properties in a complex background of other proteins that lack these properties.

1. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* Oct;17(10):994-9, 1999.

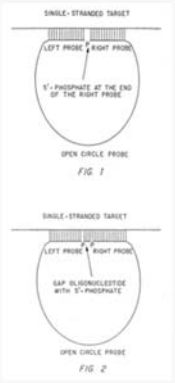
2. The seven patent applications filed include three with the title Methods for Isolating and Labeling Sample Molecules (20020168644, 20040265810, and 20040110186), Methods for High Throughput and Quantitative Proteome Analysis (20040033625), two with the title Methods for Rapid and Quantitative Proteome Analysis (20020119490 and 20060008851) and Androgen-Regulated Genes and Uses for Diagnosis, Prognosis and Treatment of Prostate Neoplastic Conditions (20040121413).

Paul M. Lizardi, Ph.D., *Yale University*

- **IMAT Award: Messenger RNA Profiling by Single Molecule Counting *(1999)***
- **Impact: Developed method to rapidly amplify an entire genome (isothermal whole genome amplification [iWGA]).**
- **Patent: Led to one patent application (Isothermal whole genome amplification kits) which are now available commercially from Amersham Biosciences and Molecular Staging Inc.**
- **PubMed: 58 abstracts; original article on rolling circle amplification (RCA) cited more than 250 times.**



KRAS exon 1 codon 12 mutations



In addition, the technology has been used and applied in a new way to fission yeast.[3] This model organism has growth mechanisms similar to those in humans. This research, led by Dr. Mark Flory in the laboratory of ICAT™ creator Dr. Aebersold, used ICAT™ and quantitative mass spectrometry to find proteins associated with centrosomes (protein structures) that accumulate in abnormal ways in cancer cells and tumors. The investigators have now developed strategies to identify related proteins in human cancer cell lines. These proteins could eventually be used as diagnostic biomarkers and therapeutic targets.

PubMed lists 218 publications on ICAT™ technology. Of these publications, 34 focus on cancer.

Rolling Circle Amplification (Yale University)

IMAT Award That Contributed to the Development of CustomSeq					
PI	**Institution**	**Title**	**IMAT Award**	**IMAT Funding**	**Dates**
Paul Lizardi	Yale University	Messenger RNA Profiling by Single Molecule Counting	R33CA081671	$1,050,685	1999-2003

Obtaining mutational profiles from clinical tissue would help stage and categorize the progression of various human cancers. However, the need for diagnostic and molecular manipulations of miniscule or fixed clinical samples has been a major limiting factor. Consequently, much effort has been applied to identifying molecular alterations in human cancers using small amounts of samples. Technologies now exist for amplifying entire genomes faithfully and rapidly from single cells and assessing thousands of genes simultaneously, which will greatly facilitate efforts to use small amounts of existing human samples in a potentially high-throughput manner.

Dr. Paul Lizardi began his work on rolling circle amplification (RCA) prior to his IMAT award, R33CA081671, and that work was published in *Nature Genetics* in 1998.[4] To date, that seminal publication has been cited 267 times. Among the specific aims of Dr. Lizardi's R33 IMAT grant was the plan to use the RCA technology to identify single nucleotide polymorphisms (SNPs) involved in early cancer development. RCA is a technique that takes advantage of the capacity of certain polymerases to use circularized ssDNA as a template. RCA can be used to robustly amplify a signal for in situ detection of fixed samples or identify SNPs. While working on the RCA technology, Dr. Lizardi developed a method to rapidly amplify an entire genome (isothermal whole-genome amplification [iWGA]). His work on iWGA led to one patent application. iWGA kits are now available commercially from Amersham Biosciences and Molecular Staging Inc.

Dr. Lizardi combined iWGA with simultaneous ligation of thousands of genes using comparative genomic hybridization array analysis to detect low-abundance mutations in cancer development.[5] This advancement has provided a potential solution for detecting the single mutant allele within a pool of "normal" tissue or cells and helps overcome smaller clinical sample availability. Furthermore, these technologies are amenable to robotic automation.

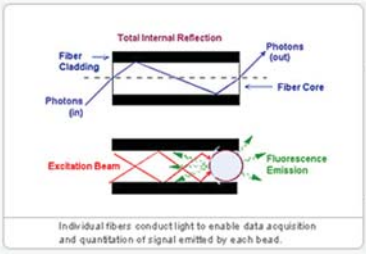
3. Flory MR, Carson AR, Muller EG, Aebersold R. An SMC-domain protein in fission yeast links telomeres to the meiotic centrosome. *Mol Cell* Nov 19;16(4):619-30, 2004.

4. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet* Jul;19(3):225-32, 1998.

5. Lage JM, Leamon JH, Pejovic T, Hamann S, Lacey M, Dillon D, Segraves R, Vossbrinck B, Gonzalez A, Pinkel D, Albertson DG, Costa J, Lizardi PM. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. *Genome Res* Feb;13(2):294-307, 2003.

Mark Chee, Ph.D., *Illumina, Inc.*

- **IMAT Awards:**
  - **Gene Expression Analysis on Randomly Ordered DNA Arrays *(1998)***
  - **Parallel Array Processor *(1998)***
  - **Protein Profiling Arrays *(1999)***
- **Impact: Ultra-high-throughput Illumina bead platform allows researchers to simultaneously assay over 100,000 points for gene expression, alternative splice detection, and protein expression.**
- **PubMed: > 35 publications relating to this technology; 6 directly relate to cancer.**



Individual fibers conduct light to enable data acquisition and quantitation of signal emitted by each bead.


Illumina's BeadStation 500 enables you to process Sentrix® Arrays at high resolution.

PubMed lists 134 articles that mention the use of rolling circle amplification in their abstracts, 9 of which are related to cancer.

Sentrix® BeadChip and BeadArray Technology (Illumina, Inc.)

IMAT Awards That Contributed to the Development of BeadChip and BeadArray Technologies and BeadLab and BeadStation 500 Processing Systems

PI	Institution	Title	IMAT Award	IMAT Funding	Dates
Mark Chee	Illumina, Inc.	Gene Expression Analysis of Randomly Ordered DNA Arrays	R43CA081952	$99,600	1999-2000
Mark Chee	Illumina, Inc.	Random Arrays for Gene Expression Profiling	R44CA081952	$976,921	2001-2003
Mark Chee	Illumina, Inc.	Parallel Array Processor	R43CA083398	$162,107	1999-2000
Mark Chee	Illumina, Inc.	Parallel Array Processor	R44CA083398	$1,027,037	2002-2004

The BeadChip microarray platform can analyze several samples on the same device at the same time. BeadChips are manufactured with very flexible microelectronic mechanical systems-based technology. This makes it possible to create any number of microfabricated wells clustered into discrete regions (arrays) on a slide-shaped substrate. They can be used for low-to-medium sample throughput requirements or dense, whole-genome applications.

Illumina's Sentrix® HumanHap500 Genotyping BeadChip makes it possible to do whole-genome genotyping of more than 555,000 tag SNP markers derived from the International HapMap Project on a single BeadChip. This BeadChip contains 555,000 tag SNP loci that offer comprehensive genomic coverage across different populations. The product can be used for whole-genome association studies and loss-of-heterozygosity/copy number studies. Because tag SNPs serve as proxies for other SNPs, this BeadChip provides higher genomic coverage with fewer SNPs than whole-genome genotyping strategies that use randomly selected SNPs.

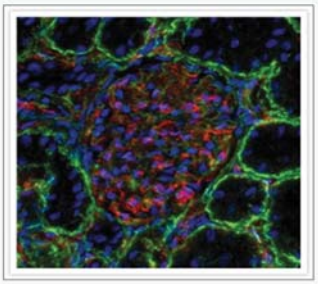
The BeadArray genotyping system combines very high throughput and accuracy with low cost per SNP analysis. The technology uses three-micron silica beads that self-assemble in microwells on fiber-optic bundles or planar silica slides. Each bead is covered with several hundred thousand oligonucleotide copies that act as the capture sequences in one of Illumina's assays. BeadArray can be used to analyze genetic variation through SNP genotyping, profile gene expression at the RNA level, and conduct proteomics research.

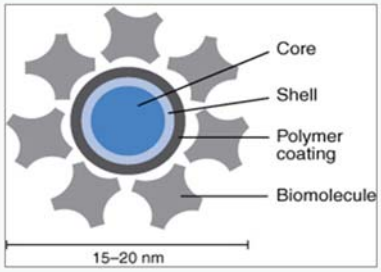
BeadArray technology is used in Illumina's Beadlab and BeadStation systems for several DNA and RNA analysis applications. The BeadLab production SNP genotyping systems can generate 800,000 to 1.3 million genotypes per day. The BeadStation enables a wide range of sample throughput and several applications for genetic analysis. The system can be used manually for both RNA- and DNA-based applications. The process can also be automated using Tecan liquid handlers and robot controls. As research needs change or new applications become available, additional applications or automation can easily be added to an existing system.

The BeadArray platforms and BeadLab and BeadStation processing systems can be used to rapidly conduct genotyping analyses on large numbers of samples, which may be used clinically to identify cancer susceptibility genes in patients. The products can also be used to rapidly evaluate the expression of genes from a whole human genome panel, which may be used to identify new molecular targets for cancer detection or therapeutic interventions.

Robert H. Daniels, Ph.D., *Quantum Dot Corp. (Invitrogen)*

- **IMAT Award: Sensitive, Multiplexed Analysis of Breast Cancer Markers *(1999)***
- **Impact: Quantum dots (semi-conductor nanocrystals) are photostable labels that emit extremely bright light in a range of colors enabling researchers to monitor complex interactions within living cells or in situ on tissue microarrays.**
- **PubMed: 38 publications on quantum dot or Qdot conjugation with streptavidin; 6 focus on use of these conjugates in cancer research.**





Two series of IMAT SBIR awards to Illumina supported the development of the bead technology. Mark Chee was the PI for both sets of awards (CA081952 and CA083398).

The goal of the Phase I IMAT R43 SBIR award (CA081952) was to develop genome-profiling analysis tools. The research funded by the Phase I award showed the feasibility of the genome-expression array and methods to determine the identity of sequences (i.e., array decoding), the development of arrays, and quality control of manufacturing. Successful completion of the research under the Phase I (R43) IMAT SBIR award resulted in the funding of a Phase II (R44) award. The Phase II research resulted in the validation of the technology's ability to accurately determine the identity of sequences (i.e., decoding), sample preparation and hybridization procedures, and validation of the products.

This support from the IMAT program resulted in the development and commercialization of the 96-well fiber-optic matrix array system and the silicon wafer BeadChip system. BeadArray became commercially available before the SBIR grant period expired, and two BeadChips were commercialized after the grant period ended—the Sentrix® Human RefSeq Genome Expression BeadChip, permitting analysis of ~24,000 genes, and the Sentrix® Whole Human Genome Expression BeadChip, permitting analysis of ~48,000 genes.

In the Parallel Array Processor awards (the R43 and R44 CA083398 awards), Illumina made progress in developing components for a new parallel array processor and protocols for genotyping, RNA profiling, and genomic DNA analyses. The overall goal of this work was to develop an automated, high-throughput genotyping and RNA profiling system. With support from the CA083398 IMAT awards, Illumina improved the software, sample handling, and detection systems and integrated these capabilities into devices. The company then evaluated these devices for performance in automatically processing large numbers of arrays. The investigators also developed procedures for RNA profiling and quantitative genomic DNA experiments.

The BeadLab system was made commercially available by the conclusion of the grant funding period. A lower cost, lower throughput genotyping station for smaller research laboratories was developed during this grant. After the grant ended, Illumina made this genotyping station, BeadStation 500, commercially available.

Illumina recently introduced a flexible, high-throughput DNA methylation profiling technology, GoldenGate®, that can be used with BeadArray to perform genome-wide methylation profiling across several areas, including cancer and human embryonic stem cell research. The first standard panel using the combined technologies covers 1,505 methylation sites over 800 cancer genes.

PubMed lists 15 publications, including one on cancer, that involve the use of BeadChip technology. An additional 18 publications report on research involving BeadArray technology. Of these, 5 address cancer research. Two more publications mention the BeadStation.

Quantum Dot Streptavidin Conjugates (Invitrogen™)

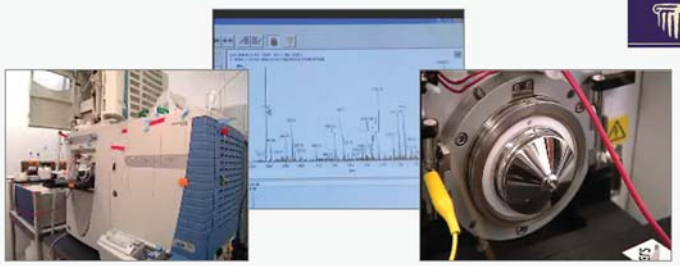
IMAT Award That Contributed to the Development of Qdot Streptavidin Conjugates

PI	Institution	Title	IMAT Award	IMAT Funding	Dates
Robert Daniels Marcel Bruchez	Quantum Dot Corporation	Sensitive, Multiplexed Analysis of Breast Cancer Markers	R44CA088391	$831,548	2000-2003

Quantum dot (Qdot) nanocrystals have strong photostability, which lets them detect low-abundance antigens. Because the nanocrystals have narrow and symmetric emission spectra, they can also be used for multicolor, multiplexed fluorescence detection using one excitation source, such as the 405-nm laser. Qdot streptavidin

John R. Yates, Ph.D., *University of Washington/Scripps*

- **IMAT Award: Direct MS Analysis of Complex Protein Mixtures *(1999)***
- **Impact: The MudPit (Multi-Dimensional Protein Identification Technology) platform marked the transition from traditional 2-D gel electrophoresis to 2-D liquid chromatography.**
- **PubMed: 97 abstracts on MudPIT, including 6 focused on cancer.**



UNIVERSITY OF WASHINGTON

THE SCRIPPS RESEARCH INSTITUTE

conjugates take advantage of the photostability of Qdot nanocrystals and streptavidin's very specific binding properties so they can be used as fluorescence detection reagents in tissue-labeling and flow-cytometry experiments. Unlike conventional dye conjugates, the Qdot streptavidin conjugates can be excited efficiently using the 405-nm violet laser, and Qdot nanocrystal fluorescence resists photobleaching.

An IMAT award contributed to the development of Qdot-protein conjugates. The goals of the IMAT award were to differentially identify multiple, spectrally distinct probes (i.e., multiplexing) in cell sections, use Qdot conjugates to characterize breast tissue sections, manufacture and characterize the Qdot conjugates, stain tissue microarrays with Qdot conjugates, and perform in situ hybridization with Qdot conjugates. By the end of the funding period, the researchers had successfully manufactured Qdot conjugates of antibodies and proteins to Qdot nanocrystals, developed staining protocols, and showed that Qdots were superior to existing conjugate probes for multiplex microscopy experiments. They also developed five Qdot streptavidin-conjugated probes.

After the funding period, the investigators developed two additional Qdot-streptavidin conjugates. All seven Qdot-streptavidin conjugates are commercially available through Invitrogen.

Streptavidin-conjugated Qdots have been used to detect Her2 cancer markers on the surface of human breast cancer cells through a biotinylated secondary antibody to human and a humanized antibody to Her2.[6] The technology has also been used to show that the N-terminal region of mortalin is involved in the in vivo and in vitro interactions of two proteins.[7] Suppression of heat shock protein 60 (HSP60) expressed by short hairpin RNA (shRNA) plasmids caused the growth arrest of cancer cells similar to that obtained by suppressing mortalin expression by ribosomes.

PubMed lists 38 publications on quantum dot or Qdot conjugation with streptavidin. Of these, 6 focus on the use of these conjugates in cancer research.

MudPIT (Multidimensional Protein Identification Technology) (Scripps Research Institute)

IMAT Award That Contributed to the Development of MudPIT

PI	Institution	Title	IMAT Award	IMAT Funding	Dates
John Yates	Scripps Research Institute	Direct MS Analysis of Complex Protein Mixtures	R33CA081665	$831,959	1999-2003

MudPIT is a non-gel technique for separating and identifying individual components of complex protein and peptide mixtures. The seminal publication on MudPIT showed the potential for applying the technology to proteomic analyses using *Saccharomyces cerevisiae* and has been cited 1,236 times since its publication.[8] Instead of using traditional two-dimensional (2D) gel electrophoresis, MudPIT separates peptides in 2D liquid chromatography. This allows greater separation of peptides that can be directly interfaced with the ion source

6. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* January 28; 307(5709):538-44, 2005.

7. Wadhwa R, Takano S, Kaur K, Aida S, Yaguchi T, Kaul Z, Hirano T, Taira K, Kaul SC. Identification and characterization of molecular interactions between mortalin/mtHsp70 and HSP60. *Biochem J* Oct 15; 391(Pt 2):185-90, 2005.

8. Washburn MP, Wolters D, Yates JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* Mar;19(3):242-7, 2001.

"Years of R01 applications on…proteomic mapping studies failed. They were deemed 'descriptive', 'fishing expedition', 'a lack of hypothesis-driven experimental design or mechanistic approach', 'overly ambitious', 'unrealistic to be achievable' and lastly, 'unlikely to be publishable'.

"I had to remove proteomic mapping studies from my PPG application before it was funded."

Jan Schnitzer, M.D.
Sidney Kimmel Cancer Center

of a mass spectrometer, which maximizes sensitivity. MudPIT avoids the band broadening associated with many chromatographic steps, which can decrease resolution.

The overall goal of the IMAT award (R33CA081665) to John Yates at the Scripps Research Institute was to develop and optimize methods for the direct analysis of individual proteins within complex mixtures. Specific aims of the research were directed at improved sample preparation, peptide separation (via multidimensional liquid chromatography), and data acquisition using tandem MS.

During the award period, solubilization of cell lysates and protein digestion conditions were improved, and 2D liquid chromatography conditions were optimized for the analysis of complex mixtures. Methods for quantification of proteins in mixtures were also developed, including the development of software tools (DTAselect and Contrast) to filter and display database search results for Shotgun proteomic applications. The software tools are available to the research community through licensing agreements with the Scripps Research Institute.

Additional applications of the technology were developed during the grant period, including studies to identify new genomic elements, the analysis of protein modifications in protein mixtures, and the identification of membrane proteins.

MudPIT has been used to identify and characterize 82 peptides in endogenous complexes that were immunoprecipitated from the androgen receptor from prostate cancer cells.[9] Information on these novel proteins could lead to better treatment or prevention interventions for advanced prostate cancer. The technology was also used to identify novel proteins released by pancreatic cancer cells involved in extracellular matrix remodeling.[10] In this study, MudPIT allowed efficient and rapid identification of proteins released by the cancer cells, including molecules that had not yet been described for the type of cancer analyzed.

PubMed lists 97 abstracts on MudPIT, including 6 focused on cancer.

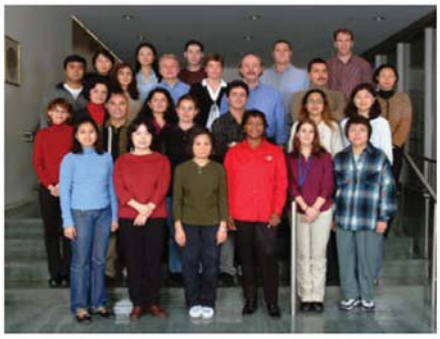
IMAT Investigators With Subsequent Awards From Selected NCI Programs

PI Name	IMAT Award(s)	Alliance for Nanotechnology in Cancer Award	Clinical Proteomic Technologies Initiative Award	Integrative Cancer Biology Program Award
James Baker University of Michigan	Photonic Crystal Fiber Probe Fluorescence Biosensing (R33 CA112141)	DNA-linked Dendrimer Nanoparticle Systems for Cancer Diagnosis and Treatment		
Xiaolian Gao University of Houston	Parallel Synthesis of RNA Oligonucleotide Microarrays (R21 CA084708)		Proteomic Phosphopeptide Chip Technology for Protein Profiling	

9. Comuzzi B, Sadar MD. Proteomic analyses to identify novel therapeutic targets for the treatment of advanced prostate cancer. *Cellscience* Jul 27;3(1):61-81, 2006.

10. Mauri P, Scarpa A, Nascimbeni AC, Benazzi L, Parmagnani E, Mafficini A, Della Peruta M, Bassi C, Miyazaki K, Sorio C. Identification of proteins released by pancreatic cancer cells by multidimensional protein identification technology: a strategy for identification of novel cancer markers. *FASEB J* Jul;19(9):1125-7, 2005. Epub 2005 May 4.

Paul Tempst, Ph.D., Current IMAT and Clinical Proteomics Awardee



"I don't believe I would have been competitive for the U24 application if it were not for the preliminary data and experience gained from the R21. So, we came out of nowhere, so to speak, and the R21/R33 clearly was the stepping stone for our U24 team."

IMAT Grant Support

R21/33 CA111942-01 Peptide Profiling Techniques to Detect Thyroid Carcinoma (2005-2007)

Impact: Addresses analytical needs that are currently not met with general profiling approaches, as exemplified in numerous surface-enhanced laser desorption/ionization (SELDI)-based publications; addresses the critical need for diagnostic approaches that can be employed in the early onset of thyroid cancer.

PI Name	IMAT Award(s)	Alliance for Nanotechnology in Cancer Award	Clinical Proteomic Technologies Initiative Award	Integrative Cancer Biology Program Award
Tim H-M Huang Ohio State University	Novel Tool for Analysis of Promoter Hypermethylation (R33 CA094441) High Throughput Methylation Analysis in Cancer (R33 CA084701)			Interrogating Epigenetic Changes in Cancer Genomes
Jan Schnitzer Sidney Kimmel Cancer Center	Technology/Map Endothelial Targets/ Human Renal Tumors (R33 CA118602) Technology to Unmask Accessible Tumor Vascular Targets (R33 CA097528)	Nanotechnology Platform for Targeting Solid Tumors		
Richard D. Smith Battelle Pacific Northwest Laboratory	Ultra-Sensitive Approach for Monitoring Gene Production (R33 CA081654) High Throughput FTICR for Molecular Analysis of Cancer (R33 CA086340)		A Proteomics Platform for Quantitative, Ultra-High Throughput, and Ultra-Sensitive	
Paul Tempst Memorial Sloan-Kettering Cancer Center	Peptide Profiling Techniques to Detect Thyroid Carcinoma (R21CA111942) Peptide Profiling Techniques to Detect Thyroid Carcinoma (R33CA111942)		CPTAC team	

IMAT Contributions to the NCI Research Portfolio

The IMAT program has pioneered the use of a grant mechanism, the R21/R33 phased application awards, for the support of innovative exploratory/developmental studies, which can rapidly move to proof-of-principle research studies if the stated milestones are met. This grant mechanism, along with the R21 and R33 grant mechanisms, are well suited for technology development, and the number of RFA grant submissions for these initiatives, based on an annual report published in 2006 by NCI's Division of Extramural Activities (DEA), has greatly expanded in the past 5 years. Based on this report, 415 technology R21/R33 and R0l grant applications were reviewed under 5 RFAs in FY 2006, representing a growth rate of 111 percent relative to FY 2002.[11] The explosive growth rate of such applications highlights both the success of the NCI in soliciting innovative technology development proposals and the increasing importance that technology development

11. Annual Report of the NCI Division of Extramural Activities, 2006.

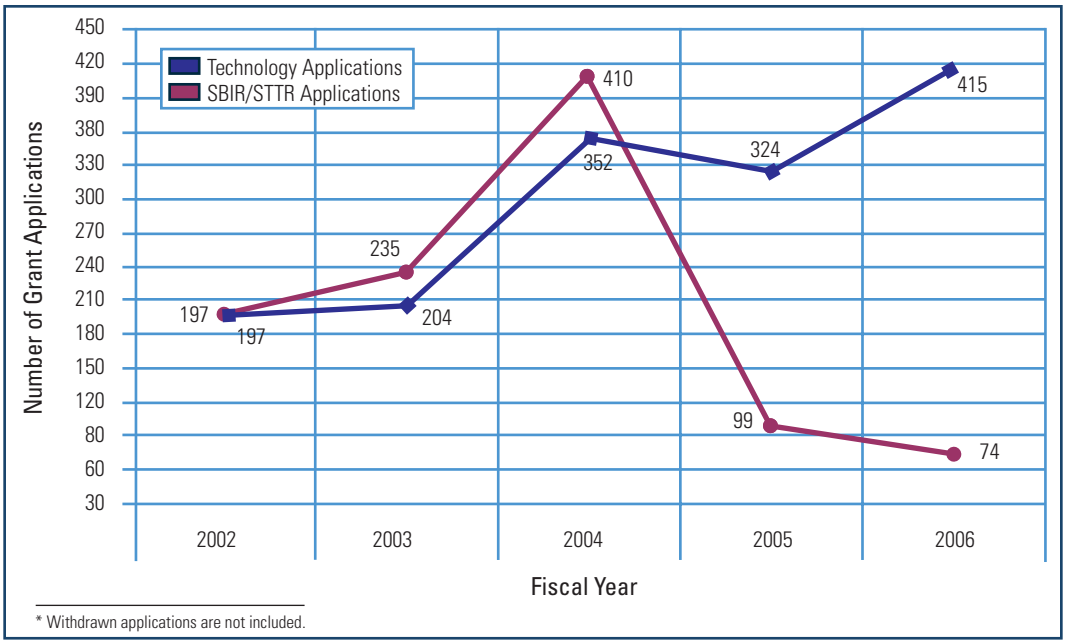


Figure 2. Technology initiatives applications reviewed* FY 2002 - FY 2006.

plays in the cancer research arena. IMAT applications constituted approximately 78 percent of all technology development applications reviewed by the NCI in FY 2005 and approximately 61 percent of those reviewed in 2006.[12] Such data indicate the strength of the IMAT program's contribution to the NCI's technology development portfolio. Between FY 2005 and FY 2006, the IMAT program awarded 95 research project grants, 26 of which represented new or first-time awardees. In addition, the program has made 51 awards in FY 2007, 5 to new or first-time awardees.

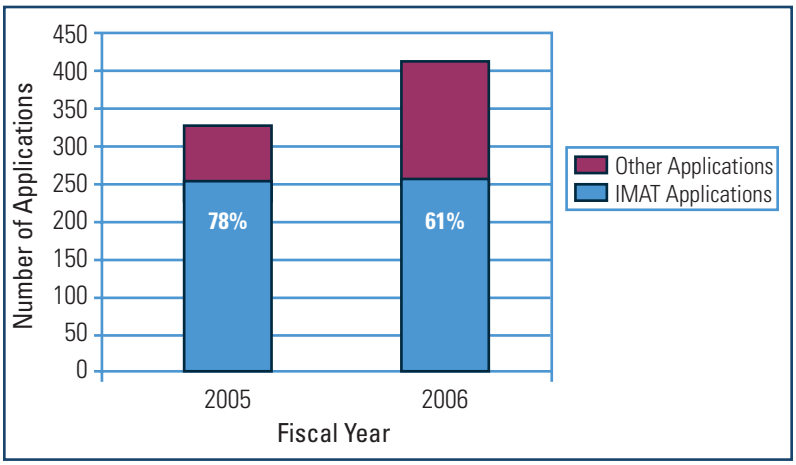


Figure 3. IMAT applications reviewed in FY 2005 and FY 2006 as a percentage of all technology development applications reviewed by the NCI.

12. Based on number of IMAT R21 and R33 applications reviewed within designated fiscal years relative to those cited by the DEA Annual Report.

IMAT Program Application Statistics FY 2005 - FY 2007		
Fiscal Year	**Total Number of Applications Received**	**Total Number of Applications Reviewed**
FY 2005	288	253
FY 2006	335	254
FY 2007	423	351

Program Progress in 2008 (January Through June 2008)

In the first 6 months of 2008 alone, since the beginning of its 5-year reauthorization period by the National Cancer Institute's (NCI's) external Board of Scientific Advisors (BSA), the IMAT program has continued to foster innovative technology development and to contribute to the NCI's overall strategic initiatives. Some of the specific contributions that continue to be made by the program are highlighted below.

Strategic and Comprehensive Portfolio Analysis to Identify Areas of Need or Otherwise Underserved Areas of the NCI Technology Development Portfolio

The IMAT program solicits and receives a wide range of applications and serves to stimulate the development of innovative technologies from inception to application and subsequent commercialization. The structure of IMAT is such that it is uniquely positioned to bridge the gap between engineers, technologists, and cancer biologists and to allow for the integration of highly innovative technology into the cancer research community. As a technology development initiative, the program has conducted an in-depth analysis of the NCI portfolio to identify technological areas of need and to better inform the development of its future funding opportunity announcements. Two specific examples of such areas identified via portfolio analysis are illustrated below.

Cellular Mechanics

Since 2003, the National Institutes of Health (NIH) has received 140 RPG applications that involve cell mechanics and has funded 51 of them, investing over $8.7 million. As the field has focused mainly on systemic fluid forces, more than 80% of the applications, and investment, involves the National Heart, Lung, and Blood Institute (NHLBI). NCI began receiving cell mechanics-related applications in 2006 but has yet to provide substantial RPG funding in this field. Of the seven applications received, four were unscored and only one (R21) received funding. Thus, this field represents an untapped area of research for the NCI, particularly with respect to the role that cellular mechanics plays in cancer metastasis and/or angiogenesis.

RPG Applications Received FY 2003 - FY 2007

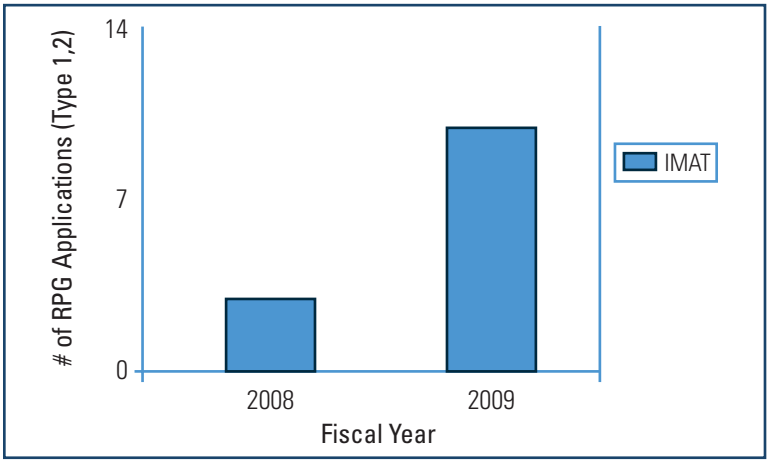
		2003	2004	2005	2006	2007
NIH	Cell Mechanics	16	25	32	31	35
	Cell Mechanics and Nanotechnology	1	1	1	3	4
	Cell Mechanics and Biospecimens	0	0	0	0	0
NCI	Cell Mechanics	0	0	0	3	6
	Cell Mechanics and Nanotechnology	0	0	0	0	1
	Cell Mechanics and Biospecimens	0	1	0	0	1

The statistics are even more disconcerting for functional cell mechanics. Since the first application received in 2004, a total of 12 applications have been received in which advanced micro- and nanotechnologies are used to quantify cell mechanics. A clear need and an opportunity thus exist for incorporating technologies from this field into the NCI portfolio.

To address this issue, the IMAT program specifically formulated and targeted this area of research and technological emphasis in its 2008 RFAs. Specific technologies solicited included the following:

- Technologies to elucidate structural modifications of macromolecules that may be indicative of or critical to the neoplastic transformation process and/or metastatic potential
- Technologies for targeted measurements made at the level of the cell, including cell-cell adhesion, cellular motility, and/or cellular adherence properties
- Technologies to quantitatively measure cytoskeletal changes and the impact of such changes on elements of metastatic potential, including increased or decreased motility, changes in intracellular mechanics, and ability of cells to interact with the environment

In response to this targeted solicitation, the number of applications submitted to the IMAT program in this emerging area of science has increased nearly threefold:



Furthermore, as of the third cycle of 2008, approximately three IMAT applications having direct relevance to the area of cellular mechanics and cancer were deemed suf-ficiently meritorious by peer review to warrant incorporation into the program's funding plan. These applications constitute the first three technology development applications funded by the NCI in this emerging area of cancer research and illustrate the success of the program in identifying, recruiting, and retaining applications from traditionally underrepresented scientific and technological areas in a relatively short span of time.

Mitochondrial Biology

Mitochondria are one of the most complex and important organelles found in eukaryotic cells. In addition to their central role in energy metabolism, mitochondria are involved in many cellular processes, and mitochondrial dysfunctions have been associated with apoptosis, aging, and a number of pathological conditions, including cancer, Parkinson's disease, diabetes mellitus, Alzheimer's disease, and cardiovascular disease.

The first patient with a mitochondrial disease was described more than 40 years ago. Yet today, for the vast majority of patients presenting with clinical signs of mitochondrial disease, the molecular cause remains unknown. Proper functioning of human mitochondria is critical to normal cellular function and metabolism, as evidenced by specific mutations in mitochondrial genes that result in severe disease phenotypes. Complicating matters, individual mitochondrial genomes (estimated at approximately 1,000 per cell) replicate continuously and can be mutated randomly, thus creating the phenomenon of heteroplasmy, in which the cell possesses more than one version of the same gene, creating a threshold effect whereby a disease might not become evident until the mutated form reaches the threshold proportion. Although much of the evidence linking mitochondrial DNA mutations and certain diseases is unequivocal, there is still a compelling need to comprehensively determine and characterize the extent to which mitochondrial DNA mutations and aberrations affect the pathology associated with diseases. In addition, comparatively few epidemiological studies of mitochondrial disorders have been conducted, and thus the full extent of the problem in general populations is unknown. Concomitantly, it is estimated that mammalian mitochondria contain approximately 1,500-2,000 proteins. With only 13 proteins encoded by the mitochondrial genome, over 99 percent of proteins in the mitochondria are encoded by the nuclear genome and subsequently transported to the mitochondria by highly regulated mechanisms. For this reason, the majority of known mitochondrial diseases are attributable to mutations in nuclear genes and are not inherited maternally. Despite the functional significance of mitochondria for cellular

and human health, a very limited number of mitochondrial proteins have been identified or characterized, and comparatively little is known about the vast majority of mitochondrial proteins in terms of their function, regulation, and relevance to human disease.

As a result, the IMAT program has identified mitochondrial research as a priority area in the NCI's strategic portfolio and is interested in transformative (high-risk, high-impact) projects that will stimulate, consolidate, and disseminate research on the molecular, cellular, genetic, epigenetic, proteomic, metabolomic, and epidemiologic mechanisms of mitochondrial disorders in an attempt to elucidate the role of mitochondrial perturbations in cardiologic, neurologic, pulmonary, vascular, cardiovascular, and oncogenic diseases.

The results of a portfolio analysis on mitochondria were used to identify mitochondriome research as an emerging area of need and potential solicitation. Such results were further used to inform the development of the Integrative Mitochondriome Project (IMP), a Roadmap concept approved by the NCI's Executive Committee and subsequently submitted to the NIH's Office of Portfolio Analysis and Strategic Initiatives (OPASI). As of July 2008, the concept was slated to be included as a specific area of emphasis within the larger NIH Roadmap through its incorporation into the transformative R01 solicitation sponsored by OPASI, illustrating the importance of this area of research and the success of ongoing portfolio analysis efforts in identifying critical areas of need.

To meet this unmet need within the NCI's scientific and technology development portfolio, the IMAT program intends to specifically target and incorporate this area of scientific research within its 2009 solicitations. It is anticipated that the technologies developed under this focus area will complement and benefit the mitochondrial research supported under both the Transformative R01 solicitation and specific strategic objectives of the Division of Cancer Control and Population Sciences (DCCPS), as reflected in its specific funding solicitations PA-08-143 and PA-08-144.

Targeted Engagement of Other NCI Strategic Committees and Initiatives

Biospecimen Coordinating Committee

Mindful of its nature as a transdivisional initiative, the IMAT program has continued to solicit input from multiple sources to better inform its continued development and evolution, thus maximizing its integration with other NCI initiatives. Before issuing its 2008 solicitations, the IMAT program solicited input from the Biospecimen Coordinating Committee (BCC) to more thoroughly inform the structure of its Innovations in Cancer Sample Preparation RFA. As a transdivisional body consisting of multiple members from various NCI divisions, the BCC was asked to provide guidance on the types of technologies each of the divisions would like to see developed under this particular component of the program. The BCC recommendations identified distinct areas of technological need within the biospecimen and sample preparation communities, which in turn formed the basis of the 2008 Sample Preparation RFAs:

- Technologies for sample collection, processing, isolation, extraction, storage, purification, and preservation
- Technologies for the assessment and/or reversal of adverse changes in samples resulting from storage and/or the use of specific preservation methods
- Technologies for the preparation of specific types of biomolecules, fluids, tissues, or other sample types that are necessary for cancer research and/or in clinical oncology

- Technologies to determine the effects of collection, processing, and storage on specific molecular components of interest in stored specimens
- Technologies that optimize the isolation and/or purification of specific biomolecules and/or specific classes of biomolecules (e.g., phosphorylated proteins, membrane-bound proteins, etc.) and/or isolation of other defined fractions from biospecimens
- Technologies for isolation of specific classes of cells, e.g., the enrichment of exfoliated cells from biofluids or isolation of stem cells from tumor specimens
- Technologies for isolation of subcellular components, such as organelles, or subcellular structures
- Technologies for measuring and monitoring changes in the properties of biomolecules of interest and the detectability of such biomolecules in specimens exposed to diverse handling variables

Biospecimen Research Network

In March 2008, the Biospecimen Research Network held its first annual symposium on advancing cancer research through biospecimen science. A number of speakers at this symposium were investigators from the IMAT program who are engaged in the active development of technologies that are relevant to the interests and strategic objectives of the Biospecimen Research Network (BRN). Some of these individuals included:

- Lance Liotta, M.D., Ph.D., George Mason University IMAT Awardee
  "Critical Pre-Analytical Variables in the Real World of Tissue Procurement"
- Brian Balgley, Ph.D., Calibrant Biosystems IMAT Awardee
  "Proteomic Discovery in Archived Tissue Blocks"

Thus, consistent with its administrative transfer to the Office of Biorepositories and Biospecimen Research (OBBR), the IMAT program's Innovations in Cancer Sample Preparation solicitations have continued to experience greater targeted integration and dissemination.

Clinical Proteomics Technologies Initiative

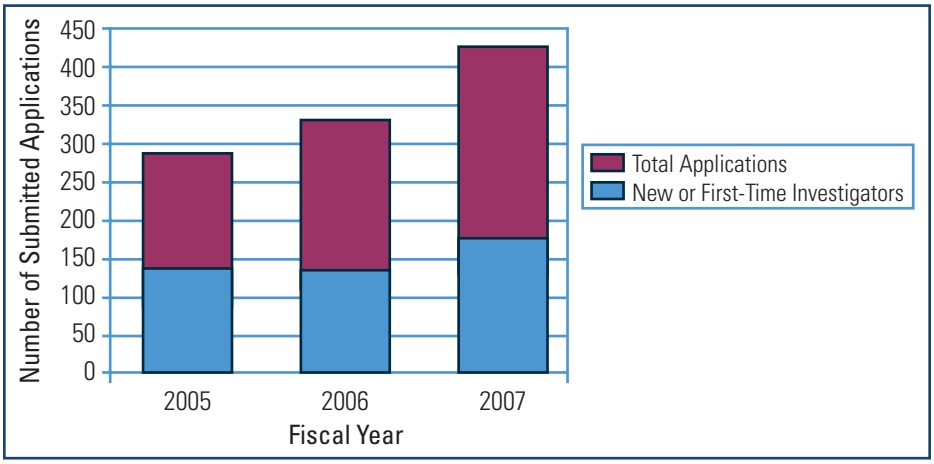
As of its 2008 solicitations, the IMAT program is actively soliciting targeted technologies in affinity capture and alternatives thereto to better serve the needs of the NCI's proteomics portfolio. In October 2008, the program is slated to hold its first-ever back-to-back Principal Investigators meeting with the NCI's Clinical Proteomics Technologies Initiative to foster cross-collaborations among different research groups and to maximally leverage the efforts of investigators from both initiatives. Among the highlights of the meeting will be a joint scientific session in which investigators from both groups will explore and highlight their mutual areas of need and potential synergies.

Emerging Technologies Continuing Umbrella of Research Experiences

Under the auspices of a potential strategic partnership currently being formed between the Center to Reduce Cancer Health Disparities (CRCHD) and the IMAT program, supplemental training programs to facilitate cross-training of biologists and technologists in cancer-relevant areas of technology development, including technologies directly pertinent to biospecimen science, will be offered to qualified IMAT investigators. It is envisioned that such programs will be used to encourage cross-collaborations in traditionally disparate fields (e.g., science and engineering, physical and biological sciences) and will target historically underserved and/or underrepresented populations. The programs will thus facilitate the bridging of the historical gap that currently exists between technology and biology (e.g., engineering/physics and cancer biologists) and provide the basis for an education and training component in innovative technology development heretofore unavailable through IMAT.

Support for New and First-Time Investigators

As part of its commitment to bring together investigators from traditionally disparate fields in order to focus their efforts on innovative cancer-relevant technology development, the IMAT program has continued to support the development of technologies from young, up-and-coming investigators. A significant percentage of applications submitted to the IMAT program in recent fiscal years have, in fact, originated from new or first-time applicants. In FY 2005, for instance, the IMAT program provided 14 awards to new or first-time investigators. Such awards were followed by 12 additional awards to new or first-time investigators in FY 2006 and 11 additional awards in 2007. As of calendar year 2008, the program continues to experience growth in both the number and quality of first-time investigators from a variety of scientific and technical disciplines. Some representative profiles follow:



Sequence Enrichment Using Droplet-Based Microfluidics

Investigator's Background. Dr. Darren R. Link is Cofounder and Vice President of Research and Development at RainDance Technologies, Inc. He received a Ph.D. degree in physics from the University of Colorado in 1998 and then continued his research on the electro-optical properties of liquid crystals as a postdoctoral scientist at Tokyo Institute of Technology. In 2001 he returned to Harvard University as a postdoctoral scientist and developed electrical techniques for manipulating droplets in microfluidic channels. This work at Harvard, in 2004, led to the founding of RainDance Technologies, a company dedicated to commercializing the use of droplets to perform bioassays in pico-liter volumes. Dr. Link has coauthored more than 55 peer-reviewed articles and 10 patent applications and is a member of the industrial advisory board for the Vanderbilt Institute for Integrative Biosystems Research & Education.

Project Summary. High-throughput sequencing of genomic DNA has gained increasing acceptance as a means to identify the genetic origins of disease. However, whole-genome sequencing of thousands of patients in large cancer studies is not yet practical. Targeted sequencing of specific exons is more practical, but there is a lack of good tools to selectively enrich target DNA sequence from a preponderance of off-target DNA. Current tools for enriching target DNA sequence result in the absence of some target regions, the inclusion of off-target sequence, and the introduction of a bias in the way target loci are enriched. These shortcomings limit the amount of information that can be obtained from sequencing samples prepared with current tools and largely offset the anticipated cost savings.

RainDance Technologies is developing a microfluidic tool for sequence enrichment that enables highly uniform amplification of target loci without the introduction of off-target sequence. The key to this technology is a library of PCR primer droplets that encapsulate individual primer pairs in 10 pico-liter droplets. These droplets are then combined with 15 pico-liter template droplets to amplify a unique target in a 25 pico-liter reaction volume using conventional laboratory thermocyclers. With processing rates of thousands of droplets per second, large numbers of individual reactions are used to amplify target loci in a simple, robust manner that is uniform across multiple loci and provides a practical, economic solution for sequence enrichment.

See article on RainDance Technologies Inc., published by *The Boston Globe* on August 31, 2008 (page 36 of this report).

IMAT Award: R21 CA 125693-01

Isolation of Circulating Tumor Cells From Blood Using Microbubbles

Investigator's Background. Dr. Dmitri Simberg received his Ph.D. degree in biochemistry from the Hebrew University of Jerusalem, Israel, where he researched the mechanisms of transfection using cationic liposomes and membranes. During his graduate studies, he developed transfection reagents that are now sold commercially. Dr. Simberg received postdoctoral training in the laboratory of Dr. Erkki Ruoslahti at the Burnham Institute on targeting tumors with nanoparticles for imaging and treatment of cancers, and additional training in the laboratory of Dr. Mattrey, where he developed targeted ultrasound contrast reagents and conceived the idea of microbubble-assisted cell separation. He currently holds a position of Project Scientist at the NanoTumor Center at University of California, San Diego.

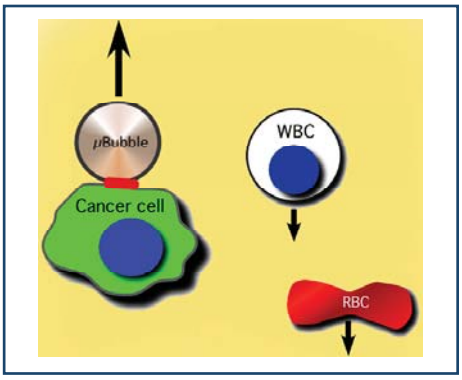
Project Summary. In cancer, malignant cells are shed into blood (1-3). These extremely rare cells (few cells/mL blood) could be isolated and analyzed to provide invaluable information for diagnosis and prognosis of cancer patients. However, because of low concentration in blood, the isolation of circulating tumor cells (CTCs) is a laborious and expensive process.

Immunomagnetic separation is the currently used method for CTC isolation from blood samples of cancer patients. This method is very sensitive (1 CTC/mL blood or lower) but produces significant contamination of nonspecific cells in the isolated sample; it is laborious and is practically limited to small volumes of sample.

In order to address the existing problems of CTC isolation from blood, Dr. Simberg and his team propose to develop a cell isolation technique based on capture of rare cells in blood by gas-filled microbubbles (μbubbles). The μbubbles coated with tumor-targeting ligands will selectively bind tumor cells in a blood sample. Because of the buoyancy of the μbubbles, they will drag bound CTCs upwards, while the rest of the cells will sediment to the bottom (see Scheme). The μbubbles will be concentrated, and tumor cells will be detected and counted.

To prove that μbubbles could selectively bind and separate the cells in whole blood, Dr. Simberg first attached a specific antibody to μbubbles in order to detect chemically modified red blood cells. Indeed, the specific antibody-coated μbubbles bound and separated the modified erythrocytes from blood. Next, he used an antibody that can recognize tumor cells; μbubbles coated with this antibody efficiently bound tumor cells that were blended with normal blood.

The ultimate goal of this project is to develop a technology for quick and efficient isolation of rare cells from large volumes of biological samples. This technology could open new possibilities for diagnostics and treatment of cancers. If successful, the technique can be used for efficient large-scale isolation of tumor cells and biomarkers as well as depletion of cells from blood and other tissues.



Scheme. Microbubble-attached tumor cells are separated from blood as a result of acquired buoyancy.

References

1. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, Meng S, Morrison L, Tucker T, Lane N, Ghadimi BM, Heselmeyer-Haddad K, Ried T, Rao C, Uhr J. *Clin Cancer Res* 8:2073-84, 2002.
2. Steeg PS. *Nat Med* 12:895-904, 2006.
3. Pantel K, Brakenhoff RH. *Nat Rev Cancer* 4:448-56, 2004.

IMAT Award: R21 CA 137721-01

Technology Development and Product Placement

RNA*Retain*

On May 14, 2008, a product based on an IMAT-funded technology (RNA*Later*) received approval from the U.S. Food and Drug Administration as a molecular diagnostic device. RNA*Retain*, a derivative of RNA*Later* technology, allows for the shipment of fresh tumor biopsies at ambient temperatures by protecting such specimens from degradation against both intracellular and extracellular ribonucleases. The commercialization and regulatory approval of this technology is but one example of the manner in which the IMAT program continues to meet its objective of fostering the development, application, and dissemination of innovative technology platforms.

Asuragen®

Asuragen Launches RNA*Retain*™, as a CE-marked In-Vitro Diagnostic Device

Austin, Texas – May 14, 2008 – Asuragen announced today the CE-mark and European launch of RNA*Retain*™ Pre-Analytical RNA Stabilization Solution, the Company's clinically validated and cGMP manufactured sample collection and RNA stabilization solution. Based upon patented technology, RNA*Retain* is labeled for the collection, storage, and transport of clinical human cellular and solid tissue specimens and stabilization of intracellular RNA within these specimens for subsequent extraction and molecular analysis.

RNA*Retain* was cleared by the U.S. Food and Drug Administration (FDA) in conjunction with Agendia BV's MammaPrint® breast cancer test in June 2007. "RNA*Retain* will become a valuable tool in molecular diagnostics and future personalized medicine applications by allowing shipment of fresh tumor biopsies at ambient temperature, thus greatly facilitating the logistical process of sample handling," said Matt Winkler, CEO/CSO, Asuragen, Inc.

RNA*Retain* infiltrates tissues and cells, precipitating nucleic acids and proteins in situ providing powerful protection of cellular RNA from both intracellular and extracellular ribonucleases, which would otherwise rapidly degrade the RNA in the specimen. Ribonucleases are ubiquitous in freshly acquired samples and need to be inactivated in order to effectively analyze the RNA composition of a specimen. The collection of human cellular and solid tissue specimens in RNA*Retain* eliminates the need to immediately process these specimens, allowing RNA extraction and molecular analysis at a later time and/or different location. It also eliminates the need to flash-freeze specimens, a process that involves manipulation of potentially hazardous agents, and to keep specimens frozen throughout storage and transport. Formalin fixation, the most common method of clinical biopsy preservation, is both hazardous to work with and is known to degrade RNA.

Lexington Company has New Analytic Device

By Davis Bushnell, Globe Correspondent
The Boston Globe, August 31, 2008

LEXINGTON - **RainDance Technologies Inc. has come up with a revolutionary technology,** the company says, that will change the way laboratory samples are analyzed for medical research and drug preparation.

"What we're essentially doing is replacing the test tube with a platform that produces [tiny] droplets at a rate of 10 million per hour," said Stephen E. Becker, 44, vice president of commercial operations. "Each droplet, the equivalent of an individual test tube, might contain a single molecule, reaction, or cell."

"We recognize that this new technology platform will change the way science is done," chief executive Christopher D. McNary, 53, said during a recent interview in the company's 27,000-square-foot facility off Hartwell Avenue, where there are 52 employees. Thirteen others will be hired by year's end, he said.

Christopher D. McNary, president of RainDance Technologies, with a fluidic circuit chip used in the DNA sequence enrichment process. (JOANNE RATHE/ GLOBE STAFF)

McNary, who previously had been a vice president and general manager of Waltham-based Thermo Fisher Scientific, said he's "unaware of any other [company or institution] using our patented technology."

RainDance's first instruments are expected to be delivered to laboratories in November, McNary said, adding that prices have yet to be announced. The instruments will be used for DNA resequencing, a major step "in detecting mutations associated with various congenital diseases," he said. "This is a $1 billion market."

Much of the technology was developed in a Harvard University laboratory run by David A. Weitz, 57, a professor of physics and applied physics. He is also a founder of the four-year-old firm along with Darren R. Link, 40, RainDance's vice president of research, and Andrew Griffiths, 44, a British scientist who is currently conducting research in Strasbourg, France.

The technology has been five years in the making.

"We took droplets of water in oil and used fluid devices to manipulate the droplets with enormous precision," Weitz said.

Besides its technology, the company has another distinction, according to McNary. "We were the first to relocate to Massachusetts in May of this year, to take advantage of " a bill passed by the Legislature that will provide $1 billion worth of grants and tax incentives over a 10-year period to the life sciences industry.

RainDance received some tax incentives and assistance from state officials in finding modern office and laboratory space, said McNary, noting that the Hartwell Avenue building had been home to MGI Pharmaceutical, which is now across the street.

The first offices of RainDance in Guilford, Conn., he said, were shared with The Rothberg Institute for Childhood Diseases. Jonathan Rothberg, 45, was one of RainDance's first investors and is now the Lexington company's chairman.

In the first half of 2007, the company received 'a little more than $30 million from venture capital firms,' McNary said. The lead firms are Mohr Davidow Ventures and Alloy Ventures, both based in Menlo Park, Calif.

Susan Siegel, a Mohr Davidow partner, said, "RainDance is enabling experiments on how the human genome can be played out." Once the stock market gyrations are over, RainDance will have an initial public offering, Siegel said. "But if the economy doesn't improve in due course, there could be another round of funding."

The Lexington firm also receives about $1 million a year from grants and collaborations, McNary said. Last month, for example, RainDance began a nonfunded collaborative effort on aging and disease with Scripps Translational Science Institute of San Diego.

"We'll explore the genetic makeup of older individuals to try to determine what predisposes some to good health and others to poor health," said Becker, the RainDance vice president.

Richard J. Roberts, 64, one of three Nobel Prize winners on RainDance's scientific advisory board, said the company's technology shows particular promise "for pushing medical diagnostics research." Roberts, who won a Nobel in 1993 for physiology or medicine, is chief scientific officer of New England Biolabs of Beverly.

The other Nobel winners are Jean-Marie Lehn (chemistry, 1987) and Aaron Klug (chemistry, 2002), who are now conducting research in France and England, respectively.

Sometime this fall, a contract will be signed with "a European country," McNary said. "A European pharmaceutical firm will also be involved in the analysis of drug compounds aimed at developing new drugs." A long-term goal in the drug-discovery field "is to develop drugs based on individuals' genetic makeup," he said.

"In the fast-emerging, so-called personalized medicine sector, the focus is on predicting and preventing disease. And we're very much a part of that movement," Becker said.

IMAT Award: R21 CA 125693-01

Information on Recently Published Patents - IMAT Funded Research Grants

The seven patents below are the most recently published patents identified through the IMAT-grant query of the U.S. Patent and Trademark Office (USPTO) Issued Patent Database. Note that all information in the tables below was directly copied from the USPTO database and not modified in any way.

Example 1. Systems and Methods for Volumetric Tissue Scanning Microscopy, Approved in 2008.

United States Patent	7,372,985
Date	May 13, 2008
Title	Systems and methods for volumetric tissue scanning microscopy
Abstract	In accordance with preferred embodiments of the present invention, a method for imaging tissue, for example, includes the steps of mounting the tissue on a computer controlled stage of a microscope, determining volumetric imaging parameters, directing at least two photons into a region of interest, scanning the region of interest across a portion of the tissue, imaging a plurality of layers of the tissue in a plurality of volumes of the tissue in the region of interest, sectioning the portion of the tissue and imaging a second plurality of layers of the tissue in a second plurality of volumes of the tissue in the region of interest, detecting a fluorescence image of the tissue due to said excitation light; and processing three-dimensional data that is collected to create a three-dimensional image of the region of interest.
Inventors	**So; Peter** (Cambridge, MA), **Engelward; Bevin** (Jamacia Plain, MA), **Ragan; Timothy** (Cambridge, MA), **Bahlmann; Karsten** (Cambridge, MA), **Kim; Ki Hean** (Cambridge, MA), **Hsu; Lily** (Arlington, MA), **Huang; Hayden** (Somerville, MA)
Assignee	**Massachusetts Institute of Technology** (Cambridge, MA)
Appl. No.:	10/642,447
Government Support	R2I/33 CA84740 from the National Institute of Health
Summary of the Invention	The systems and methods of the present invention include imaging techniques that provide quantification of morphological, biochemical and/or genetic states of cells inside tissues. Preferred embodiments of the present invention include a high-speed, two-photon, or multi-photon scanning microscope used, for example, for deep tissue imaging in highly scattering media with minimal photodamage. Real-time tissue images with submicrometer resolution in three- or two-dimensions can be obtained. A main advantage of two-photon video-rate imaging lies with its low phototoxicity. The short, pixel dwell time due to high scanning speed involves the need for optimization of the light budget. Another preferred embodiment improves on the excitation efficiency and includes compressing the laser pulse width by means of group velocity compensation and increasing the pulse repetition rate to approximate the inverse of typical fluorescence decay lifetimes. High-speed, three-dimensional (3-D) resolved two-photon microscopy provides new opportunities for the development of noninvasive biomedical applications, including optical biopsy, quantitative study of 3-D tissue architecture, and monitoring of wound healing and tissue regeneration. A method for imaging tissue, for example, includes the steps of mounting the tissue on a computer controlled stage of a microscope, determining volumetric imaging parameters, directing at least two photons into a region of interest, scanning the region of interest across a portion of the tissue, imaging a plurality of layers of the tissue in a plurality of volumes of the tissue in the region of interest, sectioning the portion of the tissue and imaging a second plurality of layers of the tissue in a second plurality of volumes of the tissue in the region of interest, detecting a fluorescence image of the tissue due to said excitation light; and processing three-dimensional data that is collected to create a three-dimensional image of the region of interest.

	The method includes a multi-photon microscope. The penetration depth of the multi-photon microscope is in the range of approximately 200-500 .mu.m. The step of sectioning further includes a microtome system integral with the microscope. The speed of the step of imaging includes at least 5 frames per second. The step of scanning further includes video rate scanning (approximately 30 frames per second). The method further includes providing a depth resolution of approximately 0.1 .mu.m or higher. In a preferred embodiment the depth resolution is between a range of 0.1 and 2 .mu.m. In accordance with another aspect of the invention, a system for providing a three-dimensional image of a region of interest, includes a light source for producing excitation light and providing at least two photons into a region of interest, a scanning microscope optically coupled to the light source, a tissue sectioning device such as, for example, but not limited to, a rotating blade, vibratome or microtome mechanically coupled to the microscope, an x-y scanner to scan the region of interest, an image sensor that detects a plurality of images of the region of interest; and a data processor that processes the plurality of images to produce a processed three-dimensional image of the region of interest. The system can include a multi-photon microscope. The microscope can be a confocal microscope. The light source is preferably a Titanium-Sapphire laser or a picosecond or femtosecond laser. The system can include a rotating polygonal mirror that provides a fast scanning axis and a galvanometer driven mirror that provides a slow scanning axis and. The system also includes a piezoelectric-driven lens translator that provides a depth axis. The system has at least one diode to generate a reference signal. The image sensor can be a charge coupled device (CCD), an avalanche photodiode or a photomultiplier tube (PMT). The excitation light is in the range of 650-1200 nm and preferably in the range of 700-1100 nm for two photon excitation. In another embodiment, a method of imaging tissue, includes the steps of mounting the tissue in a multi-photon microscope, directing at least two photons onto a region of interest, scanning a plurality of layers of the tissue in the region of interest and to limit the region of excitation, imaging a plurality of layers in the region of interest in the tissue, detecting a fluorescence image of the tissue due to said excitation light in the region of interest, processing the detected fluorescence image including the steps of sequentially storing a plurality of portions of a three-dimensional image data set, enhancing the image data set, registering individual three-dimensional data sets to generate a large three-dimensional data set, and displaying the three-dimensional data set of the region of interest. The step of processing can further include compressing the three-dimensional data set, identifying and quantifying features of the region of interest. The step of processing further includes analyzing the three-dimensional data set. The step of imaging includes, for example, imaging mitotic recombination in tissues in transgenic animals wherein recombination events give rise to a fluorescent signal. In accordance with another aspect of the present invention, a method of imaging tissue includes initially scanning a plurality of layers of tissue at a lower resolution, optically or spectrally or using a combination of both, followed by imaging a certain identified region of interest using a higher resolution imaging mode as described herein before. The foregoing and other features and advantages of the systems and methods for volumetric tissue scanning microscopy will be apparent from the following more particular description of preferred embodiments of the system and method as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

Example 2. Composite Sensor Membrane, Approved in 2008.

United States Patent	7,344,678
Date	March 18, 2008
Title	Composite sensor membrane
Abstract	A sensor may include a membrane to deflect in response to a change in surface stress, where a layer on the membrane is to couple one or more probe molecules with the membrane. The membrane may deflect when a target molecule reacts with one or more probe molecules.
Inventors	**Majumdar; Arun** (Orinda, CA), **Satyanarayana; Srinath** (Berkeley, CA), **Yue; Min** (Albany, CA)
Assignee	**The Regents of the University of California** (Oakland, CA)
Appl. No.:	**10/420,661**
Government Support	R21 CA86132-01 awarded by the National Institutes of Health/National Cancer Institute and Contract No. DE-FG03-98ER14870 awarded by the United States Department of Energy
Summary of the Invention	In general, in one aspect, a sensor may include a membrane to deflect in response to a change in surface stress. A layer on the membrane may be provided to couple one or more probe molecules with the membrane. The membrane may deflect when a target molecule reacts with one or more probe molecules. The membrane may be fixed to a substrate at a first portion and a second different portion, and may span a well in the substrate. The membrane may include a flexible material, such as a polymer. Polymers such as polyimide and parylene, or other polymers may be used. The layer may include a material to couple probe molecules to the membrane. For example, the layer may include gold. The layer may cover a portion of a first side of the membrane. The portion may be between about 5% and about 90%, or between about 10% and about 70%. A system may include a substrate and one or more membranes coupled with the substrate. For example, the system may include a membrane spanning a well, where the membrane may have a layer to couple probe molecules to the membrane. The system may also include another membrane spanning another well, where the another membrane has a layer to couple probe molecules with the membrane. The system may include a cover to enclose the well and the another well. The system may include channels to provide fluid to the membranes. In general, in another aspect, a method may include introducing fluid into a region proximate to a membrane, the fluid including one or more target molecules to be sensed. At least some of the target molecules may interact with the probe molecules and cause the membrane to deflect. The method may include measuring the deflection of the membrane. The deflection may be measured using optical detection methods and/or electrical detection methods. The details of one or more implementations are set forth in the accompanying drawings and the description below. Other features and advantages will be apparent from the description and drawings, and from the claims.

Example 3. Spectral Imaging of Deep Tissue, Approved in 2008.

United States Patent	7,321,791
Date	January 22, 2008
Title	Spectral imaging of deep tissue
Abstract	Apparatus and methods are provided for the imaging of structures in deep tissue within biological specimens, using spectral imaging to provide highly sensitive detection. By acquiring data that provide a plurality of images of the sample with different spectral weightings, along with subsequent spectral analysis, light emission from a target compound is separated from autofluorescence in the sample. With the autofluorescence reduced or eliminated, an improved measurement of the target compound is obtained.
Inventors	**Levenson; Richard** (Brighton, MA), **Cronin; Paul J.** (Charlestown, MA)
Assignee	**Cambridge Research and Instrumentation, Inc.** (Woburn, MA)
Appl. No.:	10/669,101

Example 4. Method for Determining Differences in Molecular Interactions and for Screening a Combinatorial Library, Approved in 2007.

United States Patent	7,291,456
Date	November 6, 2007
Title	Method for determining differences in molecular interactions and for screening a combinatorial library
Abstract	The invention includes a method for determining the differences between the molecular interactions of two different mixtures of molecules and identifying ligands specific for molecules in one mixture. The method utilizes a combinatorial library to compare the molecular interactions of the two mixtures and eliminates those interactions that are common to both mixtures and those that are unique to the first mixture, such that interactions essentially unique to the target mixture are identified. Ligands specific for molecules in the target mixture can then be identified. The invention also includes a method of screening a combinatorial library to distinguish between true positive beads and false positive beads and to provide for the identification of ligands specific for target molecules.
Inventors	**Lam; Kit S.** (Davis, CA), **Lehman; Alan L.** (Sacramento, CA)
Assignee	**The Regents of the University of California** (Oakland, CA)
Appl. No.:	10/057,178
Government Support	Grant Nos. R21 CA78909 and R33 CA86364, awarded by the National Institutes of Health/ National Cancer Institute
Summary of the Invention	The present invention is directed to a quick and efficient method for comparing and determining the differences between the molecular interactions of two different mixtures of molecules and of identifying ligands specific for molecules in one of the mixtures, the target mixture. The method compares the molecular interactions of the two mixtures and eliminates those interactions that are common to both mixtures and those that are unique to the first mixture, such that the interactions essentially unique to the target mixture are identified. Then, ligands specific for molecules in the target mixture can be identified. The method comprises: preparing first and second mixtures of molecules with a tag or label, where the second mixture is the target mixture; introducing the first mixture to a combinatorial library of solid phase supports; incubating the library with the first mixture of molecules; performing a first marking step to mark the solid phase supports that have molecules of the first mixture bound to them; introducing the target mixture to the library; incubating the library with the target mixture of molecules; immobilizing the library; obtaining a first image, referred to as image "A," before the marking of any solid phase supports that have

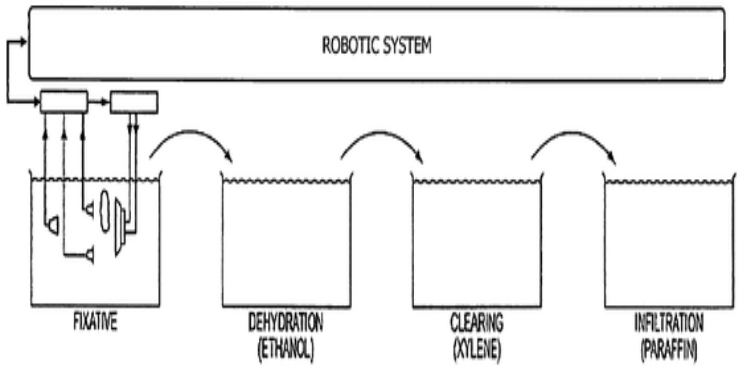
	molecules of the target mixture bound to them, showing as marked only those solid phase supports that were marked in the first marking step; performing a second marking step to mark the solid phase supports that have molecules of the target mixture bound to them; obtaining a second image, referred to as image "B," showing as marked those solid phase supports that were marked in the first marking step and those that were marked in the second marking step; performing image analysis on the first and second images to create a third image referred to as "C," showing, for each solid phase support, (B-A)/A, such that image "C" identifies the solid phase supports that were marked only in the second marking step; isolating a solid phase support identified in image "C"; and determining the chemical structure of the compound on that solid phase support. The invention is also directed to a method for screening a combinatorial bead library that quickly and accurately distinguishes between a small number of true positive beads and a large number of false positive beads and provides for the identification of ligands specific for a target molecule.

Example 5. Method for Screening Combinational Bead Library; Ligands for Cancer Cells, Approved in 2007.

United States Patent	7,262,269
Date	August 28, 2007
Title	Method for screening combinational bead library; ligands for cancer cells
Abstract	The invention includes a cell-growth-on-bead assay for screening a one-bead-one-compound combinatorial bead library to identify synthetic ligands for cell attachment and growth or proliferation of epithelial and non-epithelial cells. Cells are incubated with a compound bead library for 24 to 72-hours, allowing them to attach and grow on the beads. Those beads with cells growing are removed, and the ligand on the bead is identified. Also provided are ligands specific for cancer cells.
Inventors	**Lam; Kit S.** (Davis, CA), **Lau; Derick H.** (Gold River, CA)
Assignee	**The Regents of the University of California** (Oakland, CA)
Appl. No.:	**10/682,659**
Government Support	R33 CA89706 awarded by the National Cancer Institute and the National Institutes of Health
Summary of the Invention	The present invention is directed to a method for screening a combinatorial bead library for ligands that promote the attachment and growth or proliferation of epithelial and non-epithelial cells. The method satisfies the need for an assay that is specific and sensitive, that can be used to detect cell surface receptors susceptible to trypsin, and that can identify ligands that promote cell growth and proliferation. The method comprises introducing a suspension of live cells to a combinatorial library of small molecules, peptides, or other types of molecules, incubating the cells with the library for about 24 to 72 hours, identifying a solid phase support of the library with cells growing on the support, isolating the solid phase support, and determining the chemical structure of the compound attached to that solid phase support. The invention also includes ligands specific for cell attachment and growth or proliferation of epithelial and non-epithelial cancer cells.

Example 6. Ultrasound-Mediated High-Speed Biological Reaction and Tissue Processing, Approved in 2008.

United States Patent	7,262,022
Date	August 28, 2008
Title	Ultrasound-mediated high-speed biological reaction and tissue processing
Abstract	Methods of fixing and processing tissue and samples on a membrane by using ultrasound radiation as a part of the method are presented. Ultrasound of a frequency in the range of 0.1-50 MHz is used and the sample or tissue receives 0.1-200 W/cm2 of ultrasound intensity. The use of ultrasound allows much shorter times in the methods. Also presented are apparati comprising transducers of one or of multiple heads for producing the ultrasound radiation and further comprising a central processing unit and optionally comprising one or more sensors. Sensors can include those to measure and monitor ultrasound and temperature. This monitoring system allows one to achieve accurate and optimum tissue fixation and processing without overfixation and tissue damage. The system also allows the performance of antigen-antibody reactions or nucleic acid hybridizations to be completed in a very short time while being highly specific and with a very low or no background.
Inventors	Chu; Wei-Sing (Silver Spring, MD)
Assignee	American Registry of Pathology (Washington, DC)
Appl. No.:	09/901,013



1. A system for fixation of a tissue sample, comprising:
   - a first reaction chamber;
   - a second reaction chamber;
   - an ultrasound transducer, placeable within the reaction chambers, to irradiate a tissue sample placed within the reaction chambers;
   - a central processing unit, coupled to the ultrasound transducer, to control the ultrasound transducer; and
   - a robotic system, coupled to the central processing unit, to move the tissue sample and the ultrasound transducer from the first reaction chamber to the second reaction chamber.

Example 7. Methods for Rapid Screening of Polymorphisms, Mutations, and Methylation, Approved in 2008.

United States Patent	7,247,428
Date	July 24, 2008
Title	Methods for rapid screening of polymorphisms, mutations and methylation
Abstract	The present method is directed to methods of detecting mismatches, polymorphisms, and methylation in multiple genes or the same gene in multiple individuals.
Inventors	Makrigiorgos; Gerrasimos M. (Brookline, MA)
Assignee	Dana-Farber Cancer Institute, Inc. (Boston, MA)
Appl. No.:	10/179,053

Return on Investment: Patents Awarded Per Dollar Spent on IMAT

The number of patent applications citing the IMAT program has steadily increased since the program's inception (see Appendix 1 for patent trends). Approximately 101 patents have been filed or received by the program since 2001, with 7 additional patents approved between 2007 and the first half of 2008. All patent analyses were performed only on applications and patents in USPTO databases that specifically cited an IMAT grant number, which assumes that all inventors acknowledged this information and that they entered the grant number using 1 of 4 possible iterations in the Government Interest field. Based on these parameters, the average approximate program expenditure per patent over the course of the program's lifespan is approximately $343,353, or roughly the size of a large R21.

Conclusion

The projects described in this report show that the IMAT program is accomplishing its objectives of focusing innovative technology efforts on the cancer field and supporting highly innovative technology development projects from the scientific and medical communities. These projects have already led to numerous publications in the peer-reviewed literature, commercial applications, and patents. In addition, many of the investigators supported by IMAT have gone on to receive awards through other NCI programs, including the Alliance for Nanotechnology in Cancer, Clinical Proteomics Technologies Initiative, and Integrative Cancer Biology Program. IMAT has, in fact, contributed largely to the NCI's overall technology development portfolio. Through these accomplishments, the IMAT program is achieving its goals of accelerating the maturation of meritorious technologies from feasibility to development and/or commercialization.

With the changing landscape of technology development and the establishment of new scientific directions and paradigms, the IMAT program can continue to capitalize on its established successes through the incorporation of the following specific activities:

- Addressing the lack of awareness of IMAT among nontraditional scientific communities
- Strengthening interactions and collaborations between investigators from historically disparate disciplines to identify and address the most pressing technological needs
- Establishing a tracking mechanism by which to adequately track specific technologies through the development process
- Better engagement with specific stakeholders

As a technology development vehicle based on *investigator-initiated RPG mechanisms*, the IMAT program has continued to exhibit growth in 2008 and to sponsor a variety of highly innovative technology projects that broadly but significantly impact the cancer research arena. Through appropriate structures aimed at innovative technology development, commercialization, and dissemination, IMAT has increased the resources available to R01 investigators by providing the cancer research community with technological tools capable of significantly enhancing the quality of cancer research. Such an achievement is made even more striking by the fact that all of the accomplishments highlighted above were completed with a comparatively modest budget of approximately $10.5 million per year.

Appendix 1: IMAT Publication and Patent Trends

Purpose

To show the growth in the Innovative Molecular Analysis Technologies (IMAT) program represented as trends in publications and patent applications directly citing the IMAT grants.

Background

The IMAT Program has supported over 400 grants through 6 funding mechanisms (R21, R33, R41, R42, R43, and R44) from the program's inception in 1998 through 2007. The program continues to fund new research awards and currently has 12 open-funding opportunity announcements soliciting applications.

Methods

A list of 413 IMAT awards was compiled from multiple sources: a list developed by Macros, the Cancer Research Portfolio database, the IMAT Web site, and the Division of Cancer Diagnosis Program Web site. These awards were used to identify publications using PubMed. Queries were performed for each calendar year from 1999 to 2006 using the general format of the publication year and the actual grant numbers, for example "1999[dp] AND (CA089512[gr] OR R43CA089512[gr]…)." For the identification of patent applications, the same awards were used to query the U.S. Patent and Trademark Office (USPTO) Patent Full-Text and Full-Page Image Databases. Queries were performed using the actual grant numbers, for example, "govt/CA089512 OR govt/R43CA089512 OR govt/CA89512 OR govt/R43CA089512)."

IMAT Publication Evaluation

Significant Results

1. The number of publications citing IMAT grants markedly increased in the first 4 years of funding, after which the numbers remained steady. This result is consistent with a set of new awards that require a few years of research to be able to consistently publish results. Approximately 140 project-related publications were published in calendar year 2007, indicating a respectable level of productivity and dissemination.

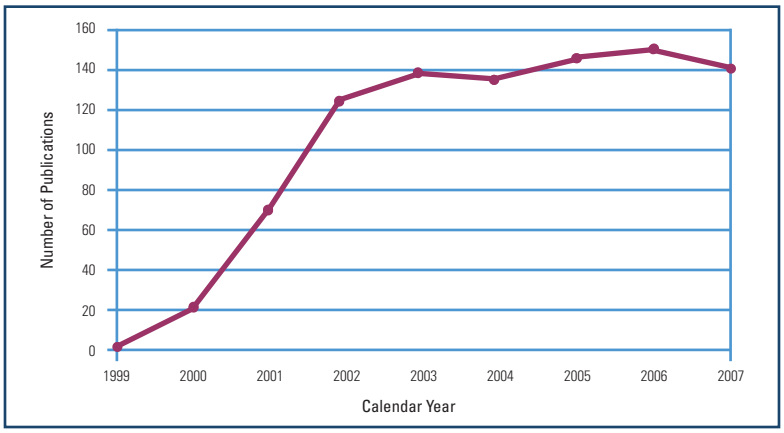


Figure 1. The trend in number of publications in peer-reviewed journals funded citing IMAT grants from 1999 through 2007.

Caveats

1. The data collection method is able to identify only publications in PubMed that cite an IMAT grant number, which assumes that all PIs are acknowledging this information to the article.

2. The data collection also assumes that all journals are publishing the grant numbers and providing them to PubMed. However, it is known that a small number of journals do not report grant numbers to PubMed.

3. These results do not differentiate the type of journal article—all types are included. For example, a review, a journal article, and a letter all could be represented in these results.

IMAT Patent Evaluation

Significant Results

1. In the first 7 months of 2008, 11 patent applications have been published by the USPTO and 3 patents have been approved.

2. For the remainder of calendar year 2007, 9 patent applications were published by the USPTO and 4 patents were approved.

3. The number of patents published that cite IMAT awards have been relatively consistent, as evidenced by both the number of patent applications submitted and patents awarded/approved over time.

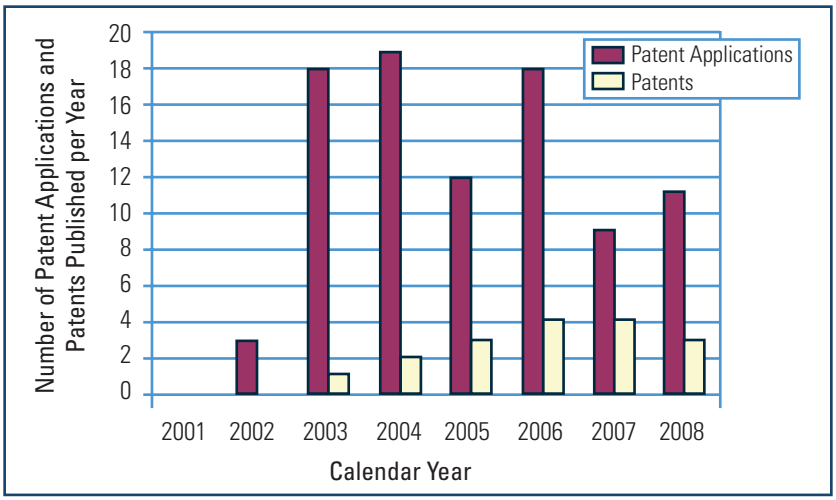


Figure 2. The trend in number of patent applications and patents published by the USPTO that cite IMAT grants in the Government Interest field from 2001 through the last 6 months of 2008.

Caveats

1. The data collection method is able to identify only those patent applications and patents in the USPTO databases that cite an IMAT grant number, which assumes that all inventors acknowledged this information and that they entered the grant number using 1 of 4 iterations in the Government Interest field.

2. The patent application office maintains records only from March 2001 to the present; therefore, patent applications published prior to 2001 will not be included in this analysis.

3. The 2008 data represent the published results through the first 7 months of 2008 only.

IMAT Grantee Name (Institution/Organization)	Number of Inventions
Allegheny-Singer Research Institute	1
Ambion, Inc.	2
Baylor College of Medicine	5
Brigham and Women's Hospital	1
Children's Hospital of Philadelphia	2
Cold Spring Harbor Laboratory	3
Columbia University, Morningside	1
Georgia State University	1
Harvard University	7
Illumina, Inc.	2
Institute for Systems Biology	4
Intronn, LLC	3
Johns Hopkins University	2
Massachusetts General Hospital	1
Massachusetts Institute of Technology	2
Medical University of South Carolina	4
Northwestern University	2
Rockefeller University	3
Sidney Kimmel Cancer Center	6
Stanford University	2
Tufts University	3
University of Arkansas Medical Sciences	10
University of California	10
University of California, Berkeley	4
University of Maryland, College Park	1
University of Missouri	1
University of Texas M.D. Anderson Cancer Center	5
University of Texas Southwest Medical Center, Dallas	5
University of Washington	5
University of Wisconsin-Madison	1
Vanderbilt University	1
Wistar Institute of Anatomy and Biology	1
Total	**101**

Figure 3. Invention count by institution (2001-2007) for organizations listed in Figure 2. Note: Information is proprietary and may not be distributed to individuals outside the NCI.

Appendix 2:
IMAT Awarded Grants (FY 2005 - FY 2007)

Introduction

This appendix has been created as a resource for viewing the scope, depth, and breadth of proposals that have been successfully funded by the IMAT program over the course of FY 2005 through FY 2007. This appendix thus contains:

- Names, affiliations, and abstracts of successful IMAT program applicants for each cycle of fiscal years 2005, 2006, and 2007,
- RFA numbers and designations to which individual proposals were made,
- Type of award associated with each successfully funded proposal (R21, R33, SBIR/STTR awards), and
- Title of each proposal.

For ease of use, the abstracts contained within this booklet have been organized by RFA title and fiscal year and in accordance with each principal investigator's last name. A brief description of the history and overall scope of the IMAT program as well as the funding mechanisms used by the program have also been provided below. The scope of each individual RFA has been provided in the pages preceding each set of abstracts. Additional information about each individual RFA may also be obtained by accessing the Office of Technology and Industrial Relations Web site at http://otir.nci.nih.gov.

The intended purpose of the IMAT program is to solicit and fund highly innovative, high-risk, cancer-relevant technology development projects in order to achieve the aforementioned objectives. The program capitalized on the success of the original IMAT program in bringing together a multidisciplinary group of scientists and engineers to work on cancer and the expansion of technology development interests across the NCI and the NIH.

Mechanisms of Support

The IMAT program utilizes R21 and R33 stand-alone mechanisms as well as the R21/33 phased innovation awards to fund high-risk, high-innovation technology platforms and approaches. IMAT program RFAs thus solicit applications under these funding mechanisms for three rounds of review each year.

In the first, or R21, phase of the program, applicants are asked to submit a grant application that includes a description of the proposed study followed by a set of quantitative milestones that are used to judge feasibility. Included in this application is a one-page description of the relevance of the technology with respect to cancer. The description may be that of a novel cancer research tool, a new detection methodology or platform, or a treatment technology that may be used either in a research setting or directly for clinical care. Reviewers are asked to review the feasibility study with an emphasis on the high-risk, high-innovation nature of the project. They are also asked to evaluate the adequacy of the milestones with respect to feasibility for the proposed technological platform in the context of the vision for its use or application but not to judge the vision itself as a long-range plan. The program staff uses reviewer comments and concerns when negotiating a final set of milestones that the applicant must meet in order to demonstrate technical feasibility and, in the case of phased innovation awards, advance to the R33 phase.

Successful applicants to Phase I of this process will receive an R21 award. The R21 award will be of modest budget and limited time (i.e., $275,000 for up to 2 years in length with no more than $200,000 being requested in a single year). To ensure that innovative technologies are able to continue toward development, funded R21 projects that prove to be feasible are eligible to apply for an R33 grant or transition. Feasibility and the R33 phase are reviewed differently from the original IMAT program. The R33 application will include the final set of negotiated milestones together with an adequate description of the completion of the milestones. This portion of the application will be followed by a thorough description of the plan for developing the technology and a description of the perceived impact the technology will have on cancer. Peer reviewers for these applications will be asked first to triage applications on the basis of completion (or lack thereof) of milestones. Those applicants found not to have demonstrated feasibility, as judged by their milestones, will be ineligible for further discussion and thus triaged from further review. Those applications that have demonstrated completion of the milestones will then be discussed by the review group with an emphasis on the development plan and the importance and timeliness of the problem to be addressed by the technology.

A brief list of the awards made by the IMAT program in FYs 2005 through 2007 follows.

Innovative Technologies for the Molecular Analysis of Cancer

Objectives and Scope

The purpose of this RFA is to encourage applications from individuals and groups interested in developing novel technologies suitable for the molecular analysis of cancers and their host environment in support of basic, clinical, and epidemiological research. Technologies to support research in the following areas are considered to be appropriate. Examples given below are not intended to be all inclusive but are illustrative of the types of capabilities that are of interest.

New tools that allow development of more complete molecular profiles of normal, precancerous, and cancerous cells, as well as the process of carcinogenesis, are needed to support the basic discovery process. The same sort of technological approaches will also be needed to examine the tumor microenvironment, including stromal and vascular interaction. These tools will also allow more thorough examination of the variations that influence predisposition to cancer and individual variability in response to therapeutic and prevention agents. Of interest are technologies and data analysis tools for:

- In vitro scanning for and identification of the sites of chromosomal aberrations that reflect inherited aberrations or somatic alterations resulting from aging, oxidation, or exposure to radiation or carcinogens, including those that are suitable for scaling for use across whole genomes, detecting DNA adducts, or detecting rare variants in mixed populations;
- In vitro scanning for and identification of sites of mutations and polymorphisms that reflect inherited aberrations or variations, or somatic alterations resulting from aging, oxidation, or exposure to radiation or carcinogens, including those that are suitable for scaling for screening whole genomes, detecting DNA adducts, or identifying infrequently represented mutations in mixed populations of DNA molecules;
- Technologies for detection and characterization of nucleic acid sequences of novel exogenous infectious agents that may be present in human cancer;
- Highly specific and sensitive detection of specific mutations;
- Detecting mismatch and recombinational DNA repair related to cancer susceptibility and drug sensitivity;
- In vitro multiplexed analysis of the expression of genes;

- In vitro detection of expression of proteins and their modified forms, including technologies suitable for expansion to profiling of all proteins expressed in cells, detecting rare variants in mixed populations, and detecting protein adducts involved in chemical mutation;
- Monitoring the function of proteins and genetic pathways, including measurement of ligand-protein complexes and technologies for monitoring protein function of all members of a class of proteins or a complete genetic pathway;
- Delineating molecular expression, function, and analysis at the cellular level in the context of both the whole body and in situ, including molecular imaging technologies suitable at this scale, contrast agents, gene amplification techniques, and related data analysis tools;
- Detection technologies and sensors, including signal-to-noise optimization and rare cell/molecule detection, of cancer and the structures and molecules important in its development and diagnosis;
- Technologies to elucidate molecular modifications of macromolecules that may be indicative of and critical to the transformation process;
- Delivery technologies and approaches to enable faster and more accurate delivery of molecular and cellular labels and drugs to and within cells for research and treatment, the overall goals being speed, accuracy, and biocompatibility; and/or
- Development of high-throughput, quantitative assays for epigenetic alterations (e.g., acetylation and methylation) in the promoter region of genes and histone proteins isolated from biological fluids and tissues.

For all technologies proposed, it will be important to substantiate the ultimate value and role of the technology in deciphering the molecular anatomy of cancer cells or analyzing the molecular profile of the individual. It is also important for applicants to discuss the ultimate potential for the transfer of ensuing technology to other laboratories or the clinic and, for more mature technologies, plans to ensure dissemination of the technology. In the case of technologies intended for use on clinical specimens or in patients, applications from or collaborations with investigators involved in the clinical research of cancer are encouraged.

FY 2005

Fluorescence In Situ Detection of Short DNA Sequences

Frank-Kamenetskii, Maxim D., Department of Biomedical Engineering, Boston University 1 R21 CA112418-01

Description: A radically new approach for the fluorescence in situ detection of DNA is proposed, which makes it possible to detect short (about 20-bp-long) single-copy DNA sequences in metaphase chromosome spreads and in interphase nuclei under nondenaturing conditions. The method of fluorescence in situ detection of short sequences (FISDOSS) to be developed will be exceedingly specific because a circular probe will be assembled via ligation of synthetic oligonucleotides on short DNA sequences opened up by specially designed peptide nucleic acids (PNAs). A high sensitivity will be provided by an efficient contamination-immune isothermal method of signal amplification: rolling-circle amplification of the assembled circular probes with incorporation of numerous fluorescently labeled nucleotides. All procedures will be performed directly on slides, and the final detection of interphase nuclei and metaphase chromosomes will be done by standard techniques using a fluorescent microscope. In Phase I, proof-of-principle experiments will be performed on arbitrarily chosen sites unique for the human genome. The goal of Phase I is to demonstrate, after initial optimization, that the short specific sequences can be effectively and specifically detected within nondenatured metaphase human chromosomes. The method will be extended to parallel multiple detections of various unique sites in the human genome. To demonstrate that FISDOSS is applicable to detect genetic markers of cancer, 12 appropriate sites associated with chronic lymphocytic leukemia will be tested. The implementation of the project will yield a convenient fluorescent in situ technique with a great potential for reliable and highly sensitive diagnosis of cancer on the DNA level.

ISSA: Novel Functional Approach to Cancer-Related Genes

Gudkov, Andrei V., Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic
1 R21 CA112586-01

Description: Identification of genes and pathways that contribute to tumorigenesis should lead to the defining of novel targets for therapeutic intervention and provide biomarkers for better diagnosis, staging, and risk assessment for individual cancer patients. Further progress in the molecular genetics of cancer would greatly benefit from a reliable methodology of assigning gene functions based on phenotypic changes resulting from modulations in gene expression. Existing techniques of this kind are based on the screening of genetically modified cells for genetic elements favoring cell growth under restrictive conditions (positive selection). Recently, a novel gene discovery methodology, named selection subtraction approach (SSA), was developed that allowed for a direct negative selection. Although proven useful for isolation of killing or growth-suppressive genetic elements, SSA capabilities are limited by the necessity to construct expression libraries. This proposal is focused on developing a new insertional selection subtraction approach (ISSA) that combines the advantages of SSA with the power of retroviral insertional mutagenesis and is based on a completely new vector system. The insertional mutagenesis arm is enhanced by the addition of a regulatable promoter, splice donor sequences, and the ability to trap polyadenylation signals. The second arm of ISSA involves "tagging" or "barcoding" each mutant (SSA), thereby allowing monitoring of the relative abundance of mutants within the population during selection by using "retrophage arrays," the key component of SSA. The ISSA technique promises to become a universal functional screening tool, free from major drawbacks of its precursors. After "technical" testing of ISSA, its power will be determined by identification of genes involved in regulation of cell sensitivity to TNF, a well-characterized system that already has been well studied by numerous approaches, including functional selection.

Longitudinal Cancer-Specific Serum Protein Signatures

Haab, Brian B., Van Andel Research Institute 1 R21 CA112153-01

Description: Serum markers hold great promise for improving the care and treatment of cancer patients. Although many proteins have serum levels associated with various cancers, each has limited clinical usefulness when measured individually at single time points. The lack of sensitivity and specificity of current serum markers stems from heterogeneity in the baseline levels of the marker proteins and heterogeneity in the tumors and patients. A biomarker discovery strategy that accounts for the heterogeneity in people and tumors is to use individualized thresholds, based on longitudinal measurements, to precisely define abnormal levels for each individual. Previous research has shown that biomarkers defined by longitudinal measurements could have greatly improved specificity and sensitivity over current markers. No systematic study of this topic has been performed, largely because of the lack of a convenient technology for that purpose. A well-developed and validated antibody microarray technology in the laboratory of Dr. Haab now makes this exploration possible. The multiplex detection capability of the antibody microarray will allow us to test the performance improvement upon using longitudinal measurements for many different proteins and to establish the general principles that define the use of longitudinal markers. In addition, the multiplex detection capability ultimately will allow the use of combined longitudinal measurements for even further biomarker performance improvement. To test this strategy, we will evaluate the sensitivity, specificity, and time of the detection of prostate cancer recurrence using both longitudinal and single time point measurements of many different prostate cancer-related proteins in serum. This new approach addresses fundamental issues in biomarker research and should result in valuable information for a wide variety of research areas. The successful demonstration of the approach to prostate cancer diagnostics will signal its potential usefulness for all types of biomarker studies.

Profiling of Redox-Sensitive Signaling Proteins

Poole, Leslie B., Biochemistry Department, Wake Forest University School of Medicine 1 R21 CA112145-01

Description: Oxidative damage and redox signaling are important components of oncogenic cell transformation, yet the molecular details of how these phenomena impact cellular proteins remain largely uncharacterized. The goal of this proposal is to develop experimental and predictive methods for the identification and molecular analysis of proteins undergoing oxidative modifications at cysteine residues, impacting cancer-related redox signaling pathways in cells. With these efforts, we will be able to apply our new proteomics-based technology to the in situ identification of redox-sensitive signaling proteins in a multi-protein, multi-pathway, whole cell format for the first time. This will allow for investigations to proceed in both discovery- and hypothesis-driven modes to unravel the molecular details of cell signaling pathways responsive to the production of reactive oxygen species (ROS). Multiple studies have highlighted the importance of activated (low pKa) cysteinyl residues in proteins as the primary targets of oxidative modifications and have shown that hydrogen peroxide is the most important ROS involved in receptor-stimulated cell signaling. The immediate product of peroxide-linked cysteine oxidation is cysteine sulfenic acid (Cys-SOH). Therefore, the trapping of Cys-SOH upon its formation in cellular proteins using a detectable reagent will yield a sensitive and comprehensive way of locating redox-responsive cysteinyl residues in cell signaling pathways. To date, no methods exist to trap this species in a manner that is amenable to proteomics type approaches; thus, identification of Cys-SOH-containing proteins must be done on an individual, targeted basis. Therefore, we propose four specific aims to (i) create and validate fluorophore- and biotin-linked reagents reactive toward Cys-SOH based on the known sulfenic acid reagent dimedone, (ii) develop "redox-profiling" protocols, using dimedone and reagents created in Aim 1 to trap Cys-SOH in proteins as formed within cells and detect the extent and location of Cys-SOH formation in proteins involved in a particular signaling pathway, (iii) develop "active site profiling" methods using bioinformatics approaches to identify particular cysteinyl residues likely to be targets of redox modifications, and (iv) validate and apply the experimental and predictive methodologies of Specific Aims 2 and 3 as tools in a total cell protein/proteomics format to analyze protein modification during the course of cancer-relevant cell signaling events. These tools will usher in a new era of redox proteomics and enable proteome-scale studies of effects of oxidative stress and antioxidant therapies on cell pathways and signaling networks.

Novel Targeted Reagents That Modify Oncogene Expression

Wright, Daniel G., Department of Medicine, Boston University Medical Center 1 R21 CA112228-01A1

Description: A detailed understanding of the genetic basis of neoplastic diseases has emerged during recent decades that has encouraged efforts to develop genetically targeted reagents both as experimental tools and as novel anticancer drugs. Peptide nucleic acid (PNA), a DNA mimic in which the phosphate deoxyribose backbone of DNA has been replaced by a pseudopeptide polymer, first described in 1991, has attracted particular interest as a gene-targeting reagent, since it is highly stable and binds to complementary RNA and DNA with high affinity and specificity. However, because PNA resists cellular uptake, its potential usefulness as a tool for modifying gene expression in whole-animal studies or as a potential therapeutic agent has been limited. In preliminary studies, we have found that Anthrax toxin "protective antigen" (PA), the component of this microbial toxin that mediates cellular delivery, is able to transport antisense PNA oligomers into cells. To explore the feasibility and potential of using native and modified forms of Anthrax PA as vehicles for delivering PNA into cells for the purpose of altering cancer-related gene expression, we propose studies with two specific aims. First, we will define the kinetics and dose limits of RA-mediated cellular delivery of antisense PNA using PNA oligomers linked to varying polypeptide sequences derived from selected toxin protein functional domains. Stably transfected cell lines engineered to express a luciferase gene interrupted by a mutant p-globin intron-2 (SIVS2-654) with an aberrant splice site that can be blocked by antisense PNA, thereby allowing luciferase expression, will be used to detect antisense activity and effective cellular delivery of PNA. Second, we will determine whether Anthrax PA permits antisense PNA-peptide constructs to alter Bcl-xL gene expression and induce apoptosis in human cancer cell lines (e.g., PCS cells) in vitro. The potential impact of this research is substantial, not only with regard to the development of experimental tools for modulating cancer-related gene expression selectively and combinatorially in cancer cells in vitro and in vivo but also with regard to the ultimate goal of developing novel agents for cancer treatment that are both genetically targeted and cell selective.

Genetic Methods for Detecting Gap Junction Communication

Wyman, Robert J., Department of Molecular, Cellular, and Developmental Biology, Yale University 1 R21 CA111993-01

Description: There is strong evidence that gap junctional communication (GJC) is a regulator of cell proliferation and that interruption of this is one of the steps in the malignant transformations of cancer. Gap junctions occur in all animal species and in most tissues from extremely early in development: the eight-cell stage in mice, gastrulation in *Drosophila*, and the two-cell stage in nematodes. Yet gap junctions are the cell structures about which the least is known; their role in cell biology and development is still barely explored. Gap junctions are difficult to detect. The standard way to determine whether cells are GJ coupled is to inject dye into one cell and see if it spreads to neighboring cells. In vivo this requires microinjection, which limits the technique to large and unusually accessible cells. We propose to develop a molecular biological method for the in vivo detection of both enduring and transient GJC without the need for intracellular injection. In the simplest version of the technology, transgenic animals will be made with tissue-specific expression of b-galactosidase (b-gal). The intact animal will be injected with a b-gal substrate (e.g., X-gal), which is taken up by cells and is hydrolyzed to a small colored reporter molecule. b-Gal is too large to pass through gap junctions, but the reporter molecule can. Cells expressing b-gal can be detected with antibodies; any cell not expressing b-gal, but filled with the reporter color must have received its color via GJC. The technology will be validated for uniform cells of a single tissue type, different cell types in a complex tissue, in gap junctions made from a variety of GJ proteins, and in heterotypic junctions made from two different GJ proteins. Quantitative measures will be taken of in-animal and across-animal statistical reliability, extent of spread, and spatial and temporal resolution of the method. Aside from b-gal and X-gal as an enzyme-substrate pair, the method will be validated using other b-gal substrates. Another similar method will be tested using tissue-specific expression of transporters to load the presynaptic cells and a detection method for transjunctional passage. The method will be applied to tumors to assess GJ coupling between tumor cells and between tumor cells and normal cells before and after tumor induction. GJ proteins will be expressed in tumors and the method used to assess GJ coupling after expression and to determine whether tumor growth has been suppressed.

Expression Profiling of MicroRNAs With Bead Array

Lu, Chiung-Mei, Genaco Biomedical Products, Inc. 1 R43 CA112735-01 (SBIR)

Description: The aim of this SBIR application is to develop a bead-based array system for the specific detection and classification of microRNAs (miRNAs). The discovery of miRNAs represents a paradigm shift that suggests the existence of many unknown cellular function and regulation mechanisms. Already, miRNAs have been implicated in different cancers and in leukemia. However, the existing methods for studying the expression of miRNAs are labor intensive and time consuming and also lack specificity and sensitivity. A more efficient and accurate research and development tool is much in demand. We have developed a bead-based array method that integrates the xMAP technology platform with the locked nucleic acid (LNA) technology. The method, called xMAP-MP for xMAP-based miRNA profiling, uses beads coupled to a capture oligonucleotide (oligo) and a biotin-labeled detecting oligo to quantitatively detect and classify miRNA. With this method, multiple miRNAs can be studied together in one reaction. Preliminary studies have shown that the assay is highly specific and sensitive: miRNAs can be detected using only 100 ng of total RNA. This is more than 100 times more sensitive than the traditional Northern blot method. The xMAP-MP is also very efficient: Samples do not need to be labeled; hybridization takes only 30 minutes; and detection and data acquisition take only a minute. The entire procedure can be finished within an hour. Furthermore, the multiplex capability of the xMAP technology platform allows the study of up to 100 miRNAs in one assay. The proposed study has four specific aims: (1) select 20 cancer-related miRNA targets and design a multiplexed detection system for these miRNAs; (2) design internal and external controls for the assay system; (3) develop and optimize a standard assay protocol; and (4) use the prototype assay to study cancer samples.

Immunoaffinity Isolation of Phosphopeptides

Rush, John, Cell Signaling Technology, Inc. 2 R44 CA101106-02 (SBIR)

Description: Among posttranslational modifications, protein phosphorylation is particularly relevant to cancer biology and therapy. However, despite advances in proteomics, it is still difficult to pinpoint phosphorylation sites in proteins. The long-term goal of this project is to develop and commercialize a multiplexed method for isolating, identifying, and quantifying phosphorylation sites using phosphorylation-specific antibodies. This method would contribute to the development of a new generation of drugs tailored to inhibit specific protein kinases with roles in cancer by identifying new phosphorylation sites that could become targets for cancer diagnosis and treatment. During our IMAT-funded Phase I, we established the feasibility of using an immunoaffinity method to isolate phosphopeptides from complex mixtures, which were then identified by tandem mass spectrometry. In this Phase II application, we will optimize the immunoaffinity method so it can be used to analyze low-level samples, using cell numbers that are comparable to what would be available from patient samples. Once we have optimized the method and established the repertory of antibodies that can be used productively in the method, we will demonstrate the method's utility by applying it to a variety of cancer cell lines to show that the method can probe the major signal transduction networks involved in cancer and can identify oncogenic lesions. We will bring a quantitative dimension to the method by merging it with mass spectrometry methods such as SILAC and Aqua, which are needed for later biomarker discovery and biomarker assay efforts.

Imaging Transcriptional Activation of Gliomas

Chiocca, Ennio Antonio, Department of Neurological Surgery, Ohio State University 1 R21 CA114487-01

Description: Differences in the expression of several genes between normal brain and its tumors (such as glioma) can provide information useful for malignant glioma diagnosis and therapy. Gliomas arise in the brain and are characterized by heterogeneous regions of necrosis, apoptosis, proliferation, invasion, and angiogenesis. Drugs are being developed to target such phenotypes, but it is unclear what imaging or molecular correlate such drugs will use to assay responses. Recently, serial analysis of gene expression (SAGE) data for malignant glioma has become available through efforts of the Cancer Genome Anatomy Project (CGAP), and several genes have been identified that are overexpressed in glioma and not in normal

brain. Some of these genes have been postulated to correlate with a particular glioma phenotype, such as invasion or angiogenesis. We propose a set of technologies useful to translate CGAP knowledge into imaging the transcriptional activation of these genes and correlate such activation with the observed phenotypic heterogeneity in in vitro and in vivo models. We plan to combine the ability of our infectious bacterial artificial chromosome (iBAC) technology to rapidly isolate and deliver into cells large genomic fragments (up to 150 kb) with the ability of MRI and bioluminescence imaging to image gene expression. Specifically, the R21 phase of this project proposes to (1) verify that large 5' flanking regions of one glioma expressed gene (for SPARC) transcriptionally activates the MRI-imaging gene ETR and luciferase, (2) image the transcriptional activation of this region in in vitro models of glioma, and (3) image the transcriptional activation of this region in in vivo models of glioma. Transgenic mouse models have shown that large regions of 5' flanking area are best at providing complex tissue-specific and developmentally correct gene expression. A large 5' flanking region of SPARC will thus be cloned upstream of luciferase and/or ETR in the iBAC system. Imaging will be performed in in vitro and in vivo glioma models. The information obtained in this R21 will justify further grants (R33, R01) where the transcriptional activation of other genes can be imaged, thus providing a quantitative anatomic map of transcriptional activation in different heterogeneous regions of gliomas (invading, angiogenic, hypoxic, proliferating areas). This provides a baseline for assessing the effects of therapies (drugs, radiation) on these tumor phenotypes. The conceptual scheme presented herein will also be applicable to a variety of diseases for which gene profiling analyses are available.

Identification of Immune Selected Breast Cancer Antigens

Claffey, Kevin P., Department of Cell Biology, University of Connecticut School of Medicine 1 R21 CA114489-01

Description: Immune-dependent responses are selective in defining non-self or aberrant antigen presentation. Unfortunately, long-term antibody production to human cancer antigens is limited. In an innovative and novel approach, we have developed a method to utilize primary immune reactions in tumor-draining lymph nodes to provide the means to define biologically active tumor antigens originating from breast cancers. This novel approach combines a series of steps to coordinate the construction of low-complexity antibody cDNA libraries and protein production that are used to identify tumor antigens using sensitive antibody microscale "antigen-trap" assays followed by LC-MS/MS antigen identification. Tumor antigens identified can then be verified as potential tumor antigens using biochemical, immunological, and molecular methodologies. The methodologies applied are medium throughput and platform based and are designed to rapidly evaluate matched lymph node and tumor samples from the same patient. This project applies innovative technologies that demonstrate that (1) tumor-draining lymph nodes are immunoreactive to aberrant breast cancer antigens and produce antigen-dependent somatic hypermutation in proliferative B-cell germinal centers, (2) antigen-binding domains of somatic hypermutated antibodies synthesized as recombinant VH and/or VHVl/Vk ScFv proteins can specifically recognize and identify breast cancer antigens, and (3) antigens identified can be verified as diagnostic for breast cancer subphenotypes. This R21 application proposes to expand and refine our methodologies to (1) determine the diversity and effectiveness of immune selection of tumor antigens in a larger patient population, (2) expand our ability to produce antibody molecules with appropriate structure and antigen binding, and (3) develop methodologies to incorporate antibody proteins synthesized into highly sensitive assays that can screen primary cancer, histological material, and/or biological fluids necessary to evaluate the potential of antigens as diagnostic biomarkers.

Nanoscale Electrocatalytic Protein Detection

Kelley, Shana O., Chemistry Department, Boston College 1 R21 CA114135-01

Description: This proposal focuses on a novel system for the electrochemical detection of cancer-related protein targets using a nanoscale electrode platform. The assay proposed relies on an electrocatalytic process involving two transition-metal ions that reports on biomolecular complexation events. Because the reporter system responds to changes in the electrostatics of an electrode surface, it will enable the analysis of nucleic acids/protein and peptide/protein complexes. The proposed project has three specific aims: (1) electrocatalytic detection of DNA repair proteins implicated in cancer using DNA-modified electrodes, (2) optimization of electrocatalytic detection and multiplexed analysis of cellular DNA repair activities, and (3) development of a generalized electrocatalytic protein detection method using prostate cancer biomarkers. The electrocatalytic protein detection system described is advantageous because it will allow the analysis and discrimination of multiprotein complexes in addition to uncomplexed analytes and will have high selectivity and specificity. Additionally, the protein detection assays will be conducted using nanoelectrodes that will allow multiplexed detection of panels of different biomolecular targets.

Nanoparticles for Efficient Delivery to Solid Tumors

Pun, Suzie H., Bioengineering Department, University of Washington 1 R21 CA114143-01

Description: New technologies for molecular analysis of cancer identify patterns of genetic and protein expression changes that have occurred in tumorigenic cells. Application of these tools for in vivo analysis is critical for a complete understanding of metastatic cancer; sadly, such studies have been limited by the lack of effective methods for delivery to metastases. Nanoparticle formulations of these agents offer in vivo protection and concentrated tumor delivery and are therefore promising delivery entities. However, a major limitation of nanoparticles for tumor delivery is restricted interstitial transport. Here, we propose to harness forces generated by actin polymerization to propel nanoparticles within the interstitial space by energy-mediated, cell-to-cell transfer, thus resulting in more efficient nanoparticle penetration. This goal can be achieved by realizing the following aims: (1) modifying nanoparticles with ActA, a bacterial protein that initiates actin polymerization resulting in propulsive forces and optimizing formulations for motility in cytoplasmic extract; (2) achieving actin-mediated, cell-to-cell transfer of nanoparticles in cultured monolayer cells; and (3) demonstrating improved nanoparticle penetration in three-dimensional spheroid cultures. Efficient delivery systems are crucial for both research and clinical applications; thus, successful completion of this project would result in a major step toward realizing the full potential of molecular analysis, detection, and treatment of cancer.

Fluorescent Aptamers for Glycoprotein Detection

Wang, Binghe, Chemistry Department, Georgia State University 1 R21 CA113917-01

Description: Early detection helps to increase the survival rate in cancer patients. One way to achieve this is the detection and analysis of molecular signatures or biomarkers that have been correlated to cancer development and prognosis. Along this line, there is a need for the development of new technologies for the molecular analysis of various cancer markers. Such spirit is reflected in an RFA (CA05002) requesting applications on developing new "detection technologies and sensors of cancer and the structures and molecules important in its development and diagnosis," among other things. In response to this RFA, we propose this feasibility study of a new platform technology that can be used for the rapid construction of fluorescent sensors for glycoproteins. We focus on glycoproteins because numerous such proteins have been implicated in cancer development. This method is based upon (1) the power of systematic evolution of ligands by exponential enrichment method (SELEX) in search of optimal oligonucleotide aptamers that can afford high affinity and specificity recognition of the target analytes, (2) the unique ability of boronic acids to recognize diol structures present on the saccharide part of glycoproteins, and (3) our own development of several fluorescent boronic acid compounds that show very significant fluorescence intensity changes (17- to 200-fold) upon saccharide or glycoprotein binding. We hope to build synergy between the SELEX approach and the unique recognition

of glycoprotein by boronic acids in making DNA aptamer-based fluorescent sensors that (1) have high affinity and specificity for the target glycoprotein and (2) exhibit very significant fluorescence intensity changes upon binding. Specifically, the project intends to develop a method to prepare DNA aptamers modified with our fluorescent boronic acid reporter compounds. The specific aims of the projects include (1) the synthesis of fluorescent boronic acid compounds that show great fluorescence changes upon binding to saccharides, (2) incorporation of the fluorescent boronic moieties into nucleotides, (3) use of the SELEX approach for the selection of sensors with optimal specificity and affinity, and (4) validation of the sensor binding with glycoproteins in solution. For this feasibility study (R21), we have selected prostate-specific antigen (PSA) as our model glycoprotein because of its importance in cancer diagnosis and the fact that glycosylation variations distinguish between physiological and pathological PSA isoforms. Such fluorescent sensors, if developed, offer the advantage of rapid and sensitive detection, the potential for high-throughput screening, and low cost. Furthermore, the same technology, once developed, can also be used for the construction of fluorescent sensors for other cancer-related glycoproteins.

Profile Serum Proteins by Glycopeptide Capture and LC-MS

Zhang, Hui, Institute for Systems Biology 1 R21 CA114852-01

Description: Cancers develop over a period of several years and are characterized by molecular changes prior to invasion and metastasis. Development of a technology that enables screening of cancer from body fluids could permit cancer detection at early and treatable stages. It is expected that the composition of the serum proteome contains valuable information about the state of the human body in health and disease and that this information can be extracted via quantitative proteomic measurements. Suitable proteomic techniques need to be sensitive, reproducible, and robust to detect potential biomarkers below the level of highly expressed proteins, to generate datasets that are comparable between experiments and laboratories and have high throughput to support studies with sufficient statistical power. In this proposal, we will develop a method for high-throughput quantitative analysis of serum proteins. It consists of the selective isolation of the peptides that are N-linked glycosylated in the intact protein using solid-phase extraction of glycopeptides (SPEG) on a robotic workstation, the analysis of these now deglycosylated peptides by liquid chromatography mass spectrometry (LC-MS), and the comparative analysis of the resulting patterns. By focusing selectively on a few formerly N-linked glycopeptides per serum protein, the complexity of the analyte sample is significantly reduced, and the sensitivity, reproducibility, and throughput of serum proteome analysis are increased compared with the analysis of total tryptic peptides from unfractionated samples. We will explore the feasibility to identify cancer-specific serum proteins in the background of normal variation using a carcinogen-induced skin cancer mouse model. The specific aims are to (1) develop chemistries and protocols for an automatic robotic system to isolate N-linked glycopeptides from serum in a high-throughput and highly reproducible fashion, (2) develop efficient and reproducible procedures for LC-MS analyses and sequence identification of discriminatory peptides by tandem mass spectrometry, and (3) explore the feasibility of this method for the identification of distinctive serum peptides specific to cancer-bearing mice in the background of normal variations. If successful, the proposed research could subsequently be used for profiling human serum samples from cancer patients and normal individuals to identify the cancer-associated proteins in serum. The identified biomarkers will open a new paradigm for performing screening and detection of human cancer at an early stage and for clinical therapeutic management.

Advanced Technology for Assaying Cancer Drug Resistance

Lim, Mark, Ambergen, Inc. 1 R43 CA114126-01 (SBIR)

Description: Significant advances have been made in the development of a new generation of molecularly targeted cancer drugs, many of which are only now emerging from the drug development pipeline. Examples of small-molecule drugs are imatinib (Gleevec), used to treat CML, and gefinitib (Iressa), used to treat lung cancer. Other drugs, such as cetuximab (Erbitux) for colorectal cancer, are monoclonal antibodies. All of these drugs selectively modulate the activity of specific target proteins such as BCR-ABL tyrosine kinase (imatinib) and EGFR (gefitinib and cetuximab) that are critical for the proliferation and survival of cancer cells. However, clinical studies are revealing that patients often develop drug resistance due to overproduction or mutant forms of the target protein. For this reason, it is likely that future treatment with single or multiple targeted cancer drugs will require testing for preexisting or acquired resistance at the level of the target protein. The principal objective of this proposal is to develop and evaluate a sensitive and low-cost technology known as PC-SNAG for monitoring resistance, to one or more drugs, in patients at the level of individual target proteins. A key feature of PC-SNAG is the ability to rapidly isolate native/active proteins from crude biological samples using photocleavable antibodies (PC-antibodies). During Phase I, proprietary high-efficiency photocleavable linkers will be used to produce PC-antibodies immobilized on beads or other solid surfaces. The PC-antibodies are used to isolate and concentrate the target proteins from a heterogeneous biological sample. The target protein is then rapidly and gently photoreleased into solution in a native and highly pure form for functional analyses that will be evaluated to detect resistant forms of the drug target and to determine optimal drug therapy on a per patient basis. In Phase I, the technology will be tested using model kinase-directed, small-molecule and antibody drugs followed by a focus on detection of drug-resistant forms of the BCR-ABL tyrosine kinase. One goal is detection of <5% of the resistant form of the target, which is difficult to achieve using conventional DNA sequencing. In Phase II, clinical evaluation of the technology will be performed in collaboration with Dr. Daniel Write, Chief of Hematology/Oncology at the Boston University Medical Center.

Detecting Integrated HPV DNA in Immunocompromised Women

Moen, Phillip, One Cell Systems, Inc. 1 R43 CA113926-01 (SBIR)

Description: Human papillomavirus (HPV) infection is the cause of virtually all genital warts (condyloma), and cervical and anal cancers. The company has developed a novel assay for detecting integrated HPV DNA in cervical cells, which has the potential to more accurately predict disease progression and prognosis. Using this novel assay format on cervical cell samples from HIV-positive women, this SBIR will investigate the HPV types involved and whether HPV DNA has integrated into the host genome. A rapid cell-based in situ hybridization assay for screening cervical cell samples from HIV-infected women is urgently needed because conventional HPV diagnostic methods such as simply determining whether an infection involves high-risk or low-risk families are not as useful in this patient population. In immunocompetent women, most infections, regardless of viral type, resolve spontaneously over several months. HPV prevalence in asymptomatic immunocompetent women ranges from 10 percent to 20 percent and, in most cases, involves infection by only a single viral type. In contrast, typically >70 percent of HIV-positive women have HPV infection, which results in cervical and anal cell abnormalities. Viral persistence is greatly increased, as is the frequency of multiple type infection. Higher relapse rates after condyloma treatment are seen, as well as differences in HPV strain prevalence.

Photonic Crystal Fiber Probe Fluorescence Biosensing

Baker, James R., The Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor 1 R33 CA112141-01A1

Description: Methods using fluorescent probes to identify cancer signatures and biological activities of cancer cells hold great promise. Probes based on fluorescence resonance energy transfer (FRET) and techniques such as fluorescence correlation spectroscopy (FCS) and other similar technologies offer the ability to identify specific RNA or protein molecules that can identify a cancer and provide information on oncogenic pathways used by the tumor cells. Other probes can give insight into drug response by measuring apoptosis induction by chemotherapies and radiation. However, fluorescent analysis has several limitations. Most ex vivo analyses use a flow cytometer or complex, confocal microscope to perform analyses, and this requires that tissue be removed from the body and often disrupted into cells, then fixed and analyzed in a static manner. The problems with in vivo fluorescent analysis are even greater since background fluorescence and tissue scattering, even in the near-infrared range, limit signal acquisition to the skin. Two-photon excitation has been a critical advance in optics, facilitating FRET, FCS, and CARS techniques in vitro. However, these applications are limited by the complex technology (confocal microscopy) necessary to employ these techniques. We have demonstrated the use of two-photon fluorescence analysis through optical fibers for analysis of cancer cells in vitro and human tumors in vivo in SCID mice. This prior work constitutes the equivalent of an R21 proposal, as we achieved our major objectives: to develop sensing system optics and electronics and to document the ability of this system to obtain and analyze fluorescence signals in vitro and in vivo. The primary goal of this R33 application is to develop a more sensitive prototype device based on a novel dual-clad photonic crystal fiber (DCPCF) that we hypothesize will provide the sensitivity and redundancy necessary for the clinical evaluation of fluorescence signals in vivo using several fluorescence techniques. We plan to carry out our studies in three Specific Aims: (1) develop DCPCF for use in a two-photon optical fiber fluorescence probe (D-TPOFF), (2) utilize D-TPOFF to quantify cancer signatures in vitro and monitor drug effects in tumor cells using targeted nanoparticles ex vivo and in vivo, and (3) utilize D-TPOFF to adapt other fluorescent techniques to examine events in tumors in vivo. At the end of these studies, this technology will be at a point where it is ready for commercialization.

Transcription Factor Reporter Technology

Bogdanov, Alexei A., Radiology Department, University of Massachusetts Medical School 1 R21 CA116144-01

Description: Molecular dissection of gene expression pathways in cancer has revealed many new targets for cancer therapy. Those targets include the components of abnormal transcription machinery. Proteins involved in regulation of transcription attained high priority because of the convergence of many signal transduction pathways at the transcriptional level. New molecular therapies directed to transcriptional targets have significant advantages over traditional therapies because of the precision of their interference with target gene expression. Consequently, small-molecule inhibitors, DNA binding polyamides, protein-binding oligonucleotide decoys, as well as small interfering RNAs and their combinations are being developed for cancer therapy. While rapid progress in molecular genetics and medicinal chemistry delivers new "attenuators" of gene expression, the technologies of early and noninvasive assessment of cancers that would be amenable to these therapies are currently lacking. In particular, imaging technologies that report directly on gene transcription in cancer cells are critically important for both cancer phenotyping and staging, as well as for evaluating new therapies. The goal of the proposed research is to optimize and characterize far-red fluorochrome-labeled oligodeoxyribonucleotide molecular reporter (ODMR) probes followed by the investigation of their transcription-factor reporting properties. Using NF-κB as a model cancer-relevant transcription factor, the following specific aims will be pursued: (1) optimization of design, synthesis, and in vitro testing of transcriptional factor reporter probes (ODMR) and (2) investigation of the potential of ODMR probes in detecting active transcription factor in live cells.

Transducing Tumor Cell Antigens to Amplicons

Ellington, Andrew D., Department of Chemistry and Biochemistry, University of Texas at Austin
1 R21 CA107887-01A1

Description: There are numerous methods for identifying and probing genomic markers in tumor cells, most of which are based on nucleic acid amplification technologies. However, in many instances protein markers are going to be of even greater utility in identifying and classifying tumor cells than genetic markers. To this end, it would be extremely useful to have methods by which protein (rather than nucleic acid) markers could be amplified. In this proposal we outline a series of novel methods for transducing tumor cell antigens to amplicons, which in turn can be sensitively detected using methods common to nucleic acid diagnostics. In particular, it has previously been shown that nucleic acid binding species (aptamers) selected from random sequence pools can specifically interact with a wide range of protein targets, including those relevant to tumor biology. Aptamers typically bind their cognate targets with dissociation constants in the nanomolar range and can readily discriminate between even closely related proteins. For these reasons, aptamers should prove useful for recognizing a wide range of protein markers associated with cellular transformation. We have previously developed automated methods for the selection of aptamers. We now propose to use such automated methods to target cell surface antigens of tumor cells. Selected antitumor aptamers will be adapted to a number of important diagnostic methods, including methods to label tumor cells and methods to transduce tumor protein antigens into nucleic acid amplification assays, via two novel methods—proximity ligation assays and rolling circle amplification.

Automated Glycoanalysis of Cancer-Related Proteins

Pannell, Lewis Kenneth, Cancer Research Institute, University of South Alabama 1 R21 CA116070-01

Description: The quote that "aberrant glycosylation is the hallmark of cancer cells" is reflected in numerous reports in the literature documenting changes in glycosylation on specific membrane proteins in cancer cells relative to normal cells. These changes have been shown to be involved in the release of cancer cells into the extracellular matrix and in the formation of metastasis. Glycosylated proteins represent a huge, almost untapped source of biomarkers, considering the wealth of evidence documenting their significance in cancer. Unique glycoforms could be used for diagnostic purposes, to target drugs at cancer cells, and for the development of immunotherapy. Despite the evolution of new mass spectrometry-based methods for protein analysis, few of these involve the determination of posttranslational modifications, especially glycosylation. As routine methods (e.g., MS/MS-based sequencing methods) yield little light on glycosylated peptides, this proteomics research facility has established a new approach to automatically identify glycan structures on pure proteins from commercial or recombinant sources. It involves the acquisition of molecular weight only spectra and the detection of the glycosylation patterns using accurately determined mass gaps between the various glycoforms. The presence of multiple glycoforms is used to enhance the analysis rather than to confuse it. The approach has been shown to be reliable and extremely fast (taking less than 1 second) at identifying and characterizing such sites, including in proteins with highly complex glycosylation patterns. The aim of this proposal is to prove its utility in cancer where changes in glycosylation are interlaced with the progression of the disease. It will concentrate on both the cell surface proteins and those secreted from cells. Data will be compared to previously published reports where available. The glycans on previously uncharacterized proteins will be established and validated against the best hand-interpreted results. The long-term aim is to make glycoanalyses routine to all cancer investigators, and the software integral to the approach will be made publicly available on a Web site. This will represent the first step, with the glycoanalysis of full proteomes being an ultimate objective.

Single-Molecule Genome Analysis of Oligodendroglioma

Schwartz, David C., University of Wisconsin, Madison 1 R33 CA111933-01A1

Description: The proposed aims of this project center on constructing whole-genome maps from 30 different oligodendroglioma tumor samples, a solid tumor that has confounded conventional genome analysis approaches to associate loss of heterozygosity (LOH) with a distinct set of gene(s). The research proposed reflects a multidisciplinary collaborative effort to use a robust single-molecule platform (Optical Mapping) to construct high-resolution restriction maps from a selected group of characterized tumors bearing a heterogeneous genome population. Chromosomal aberrations will be scored and classified on a whole-genome basis in the absence of any hypothesis, outside of the established link between 1p/19q LOH and diagnostic purposes. Genomic aberrations in the tumor samples—deletions, insertions, translocations, tandem amplifications, and gross rearrangements—will be precisely located and characterized. Map coverage of 20-50x will ensure discernment of separate populations of chromosomal aberrations within each sample at 50-500 kb genome intervals. New algorithms will be developed, based on Optical Mapping data, to identify breakpoints within a heterogeneous population of aberrant genomes based on the local alignment of single-molecule barcodes, or Optical Maps, with the latest build of the human genome sequence. Aberrations will be statistically assessed to discern the percentage of the tumor cell population bearing a given genomic lesion. To synergize this, a new generation of microfluidic device to incorporate cell lysis and DNA loading within the same disposable silicone fabrication will be perfected. These first-ever whole-genome maps of oligodendroglioma tumor genomes and comprehensive determinations of aberrations will be entered into a customized Santa Cruz Genome Browser as additional annotation tracks. This technology provides a unique platform to decipher the complex molecular anatomy of cancer cells, on a whole-genome basis, at high resolution.

Genome Instability in Cells and Tissues of the Zebrafish

Spencer, Forrest A., Department of Biochemistry, Cellular, and Molecular Biology, Johns Hopkins University 1 R21 CA116210-01

Description: Change in genome structure can occur in mitotic and meiotic cell lineages, and this contributes to individual variation, evolution, and disease. It has been argued that stochastic somatic genome instability makes an early and important contribution to the development of human cancer, largely through loss of wild-type tumor suppressor genes. Many tumor suppressor genes themselves are guardians of genome structure and proper cell cycle control, and their loss may cause additional somatic instability. Thus, high levels of genomic instability may be viewed as possible cause and/or effect of steps in tumorigenesis. To distinguish these, a method is needed for continuous monitoring of genome stability in cell lineages that give rise to cancer. Genome stability in vertebrates is currently followed using karyotype analysis, fluorescence in situ hybridization, or measurement of endogenous marker loss using cell selection procedures. At this time, an in vivo system that can be used to determine genome stability in situ (i.e., without tissue disruption) is lacking. We propose the zebrafish *Danio rerio* as an ideal model system in which to develop this view of vertebrate biology, and we outline a novel method for following marker stability in fish. The method is based on a transcriptional repression design in which repressor loss leads to expression of the fluorescent protein EGFP. Using transgenic zebrafish, we will perform proof-of-concept tests for detection of repressor loss and characterization of repressor loss mechanisms. The zebrafish is ideally suited for development of this strategy due to the ease of organ visualization and its well-developed genetics and genomics tools. Furthermore, the rapid generation time and small size of the zebrafish support cost-effective observation of many individuals, providing statistical power. In future work, this measurement of genome stability in situ will be important for understanding the relationship between gene function and genome instability in different tissues and between genome instability and tumor development.

In Vivo Gene Detection for Cancer Analysis

Bao, Gang, Institutional Partner: Vivonetics, Inc. Academic Affiliation: Department of Biomedical Engineering, Georgia Tech 2 R42 CA103103-02 (STTR)

Description: We propose to develop a novel dual FRET molecular beacons technology for living cell detection and analysis of cancer. Molecular beacons are dual-labeled oligonucleotide probes with a stem-loop hairpin structure. Hybridization of molecular beacons with target mRNAs corresponding to cancer markers results in fluorescence of the cell. Thus, cancer cells (bright) can be distinguished from normal cells (dark). However, the conventional design of molecular beacons may induce a significant amount of false positives in cancer cell detection due to probe degradation by nucleases and nonspecific interactions. To overcome this difficulty, we have developed the dual FRET molecular beacons approach in which a pair of molecular beacons with respectively donor and acceptor fluorophores hybridizes to adjacent regions of the same target mRNA and results in a FRET signal upon proper excitation, which is readily differentiated from non-FRET false-positive signals due to probe degradation and nonspecific probe opening. In our Phase I STTR studies, we have demonstrated that, using dual FRET molecular beacons in living cell mRNA detection, false-positive signals can be significantly reduced. We have also developed new molecular beacon delivery methods with high efficiency and fast kinetics for live-cell studies and examined the sensitivity and specificity of detecting Kras and survivin mRNAs in living cells. In Phase II STTR studies, we will demonstrate the quantitative capability of molecular beacons in detecting and analyzing cancer genes in living cells. We will use dual FRET molecular beacons to detect the upregulation of specific genes and compare the mRNA levels detected using molecular beacons and RT-PCR. To further increase the detection sensitivity and specificity, we will use molecular beacons to target multiple sites on the same mRNA molecule and target multiple tumor markers in the same pancreatic cancer cells. We will demonstrate the capability of molecular beacons to detect mutant mRNAs in fixed or live cells and the sensitivity of detecting a small number of cancer cells in a sample. The goals are to develop the dual FRET molecular beacons technology for early cancer detection and diagnosis and to commercialize this technology for a wide range of biomedical applications, including cancer analysis, drug discovery, and in vivo detection of gene expression in basic biological studies.

Multiplexed Protein Biochip Assays With Signal Amplification

Kim, Raymond, Geneprism, Inc. 1 R43 CA112612-01A1 (SBIR)

Description: Technical innovation has transformed our ability to analyze genetic information in a comprehensive fashion. DNA biochips and related technologies now permit the simultaneous measurement of the structures and activities of essentially all human genes. This comprehensive capability has given us our first glimpse of the complexity of the underlying molecular events that define metabolism and disease pathogenesis. To complement this information and translate its findings to the diagnosis and treatment of human disease, these observations must be translated and extended by direct measurement of the proteins that are encoded by this genetic information. Within the pharmaceutical, biotechnology, and research communities, there is currently a large unmet need for multiplexed protein detection and quantification technologies. The current techniques for multiplexed protein profiling rely heavily on application of sandwich-ELISA format in miniaturized scale. However, these techniques suffer limited multiplexing capabilities and variable performance, including specificity, sensitivity, and accuracy. The innovation described in this proposal is designed to alleviate these limitations by eliminating the need for the use of sandwich-ELISA in microarray assays and introducing signal amplification mechanism to improve the assay performance. This innovation, termed "protein footprint scanning technology," is founded on a novel immunochemical detection method that combines the specificity of antibodies with regiospecific amino acid cross-linking chemistry to produce analyte-specific quantification. The successful validation and implementation of this technology will catalyze the development of highly sensitive protein microarray assays using only one antibody per target analyte. This will in turn enable microarrays with higher content multiplexity, diversity, and novelty. The expanded content will be especially important for cancer research since the multifactorial nature of oncogenesis will likely require parallel examination of large numbers of proteins in cellular and extracellular proteome compartments. As a proof of concept, the feasibility of applying this innovative technology for protein detection and quantification will be validated using multiplexed protein microarray assays consisting of 10 validated cancer biomarkers.

FY 2006

Tadpole Assays for the Molecular Assessment of Cancer

Brent, Roger, The Molecular Science Institute 1 R33 CA114306-01A1

Description: Biologists have long known that cancer cells sometimes announce their presence by shedding certain molecules into the blood. More recently, many have come to believe that some cancers might be detectable by "signatures," patterns of sets of molecules, perhaps normally present in the blood, but for certain cancers, present in higher or lower numbers than normal. Recently, we learned to make new kinds of molecules, which we call "tadpoles." We have demonstrated that we can use them to detect and count small numbers of molecules. These assays are relatively simple and relatively inexpensive, and they should be applicable to both kinds of cancer detection. During the next 3 years, we seek funding to develop and "harden" these assays to the point that they can be tested in clinical cancer diagnosis.

An RNA Sensor for Detection of Circulating Tumor Cells

Clawson, Gary, Department of Pathology, Biochemistry, and Molecular Biology, Jake Gittlen Cancer Research Center, Pennsylvania State University 1 R33 CA118591-01

Description: This proposal seeks to develop an RNA Sensor to be employed for detection of circulating tumor cells. RNA detection is based upon a hybridization "sandwich." Two target RNAs have been chosen for clinically important cancers (prostate, breast, and melanoma), and library selection protocols will be utilized to identify/optimize accessible sites for antisense oligonucleotide (ASO) binding. Silicon nanowires will then be covalently derivatized with ASO to a library-selected site (ASO-) in the target RNA. The ASOi nanowires will then be deposited by fluidic deposition onto chips and integrated into the underlying CMOS circuitry. Target RNA will be purified from cellular preparations and will then be hybridized to the ASd-nanowires. An ASO2, targeted to a second library-selected site, will be covalently attached to 12 nm gold particles (ASO2-nanoprobe). Binding of the ASO2-nanoprobe to the target RNA-ASOi-nanowire complexes will induce a resonance frequency shift in the nanowires, which is greatly amplified by the mass of the gold particle. This resonance frequency shift (R??) will be detected by direct electrical read-out, with voltage (quantitatively) related to binding events (R??) initially detected optically. We have successfully measured R? of 300 nm silicon nanowires (with high Quality-Factors) under ambient conditions. Theoretical calculations predict very good Quality-Factors for silicon nanowires in H20, and detection of single binding events should be achievable.

Preliminary data related to all aspects of RNA Sensor development have been obtained. These include library selection of target sites in prostatic DD3 RNA, sandwich hybridization specificity "off-chip" synthesis and derivatization of nanowires, R? measurements with nanowires, and fluidic deposition of nanowires on chips. After basic developmental steps are completed, experiments will include quantitative determination of target RNAs using the detection device compared to QPCR amplification. The Specific Aims for this funding period are designed to develop an RNA Sensor appropriate for subsequent use in clinical validation studies for circulating tumor cells. Successful development of this RNA Sensor would provide a major advantage over PCR-based assays and could form the basis for high-throughput screening tests for simultaneous detection of many different circulating tumor cell types.

Microarray Platform for Profiling Cancer Proteases

Crabatt, Benjamin, Department of Cell Biology, The Scripps Research Institute 1 R33 CA118696-01

Description: Proteases are suspected to play major roles in cancer, including the activation/inactivation of growth factors and the degradation of extracellular matrix components to promote cancer cell migration and invasion. Consistent with this premise, transcript and protein levels for many proteases are upregulated in cancer cell lines and primary tumors. However, whether these changes in protease expression correlate with changes in protease activity remains a critical, but largely unanswered, question. Indeed, proteases are regulated by a complex series of posttranslational events, meaning that their expression levels, as measured by conventional genomic and proteomic methods, may fail to accurately report on the activity of these enzymes.

To address this important problem, we have introduced a chemical proteomics technology referred to as "activity-based protein profiling (ABPP)," which utilizes active site-directed probes to determine the functional state of large numbers of proteases directly in whole-cell, tissue, and fluid samples. We have applied ABPP to identify several protease activities upregulated in human cancer cells and primary tumors. Recently, we have created an advanced antibody-based microarray platform for ABPP that enables profiling of protease activities with an unprecedented combination of sensitivity, resolution, and throughput, while requiring only minute quantities of proteome. The goal of this R33 application is to extend these studies to create the first ABPP microarray for the parallel analysis of key cancer-associated protease activities in any proteomic sample. These studies will deliver valuable new reagents and methods for the functional characterization of proteases that will be made freely available to the cancer research community. We envision that the general implementation of these innovative technologies will greatly accelerate the discovery of proteases with altered activity in human cancer. These proteases may in turn represent valuable new markers and targets for the diagnosis and treatment of cancer.

Transposon-Based Somatic Mutagenesis/Prostate Cancer Genetics

Largaespada, David A., Academic Health Center, University of Minnesota Cancer Center 1 R21 CA118600-01

Description: Prostate cancer is the second leading cause of male cancer death in the United States and often results in a reduced quality of life for those living with or being treated for this disease. Prostate cancer is commonly treated by androgen ablation therapy, and although many tumors initially respond to this treatment, many eventually progress to hormone refractory prostate cancer (HRPC). The genetic basis for the transition to hormone insensitivity is poorly understood. We propose to use a mouse model for invasive prostate cancer that results from prostate-specific loss of the tumor suppressor gene Pten. This mouse model is relevant to human disease as Pten expression is lost in many human prostate tumors and the tumors that form in the mice remain partially sensitive to hormone withdrawal. We will use a novel method for cancer gene discovery in mice, the Sleeping Beauty (SB) transposon system, to promote aggressive tumor formation in this model. The SB transposon is a DMA element that is capable of mobilizing and inserting in a different location in the genome. If a mobilized transposon reinserts near a cancer gene, it can promote changes in expression of that gene that promotes the transition from a normal cell to a transformed cancer cell. We have previously generated mice engineered with all the components necessary for mobilizing SB transposons in various tissues in the adult mouse. In unpublished experiments, we have successfully used the SB system to identify genes involved in sarcoma and lymphoma formation in mice, and we believe that SB will prove to be equally as successful in prostate tumor models. By using SB to promote HRPC formation, we can both identify the genetic changes that cause a tumor to become insensitive to hormone withdrawal and also generate a useful mouse model of HRPC that will be useful for discovery and testing of novel chemotherapeutic agents for advanced prostate cancer. Finally, this approach represents a novel method for the unbiased molecular/genetic analysis of cancer development and could be used widely in the study of important clinical cancer problems.

DXMS-Facilitated Membrane Protein Construct Design/Cancer

Woods, Virgil, Department of Medicine, University of California, San Diego 1 R21 CA118595-01

Description: Many cancer-implicated proteins are integral membrane proteins (IMPs). There is a pressing need for improved methods for the production of IMP constructs for use in high-resolution structure determination efforts. Three years ago, we completed a 15-year effort to develop methods for the performance of peptide amide hydrogen/deuterium exchange-mass spectrometry (DXMS). In collaboration with the Joint Center for Structural Genomics (JCSG), we recently demonstrated that DXMS can provide precisely the information needed to guide the design of well-crystallizing constructs of otherwise poorly crystallizing soluble proteins. The NCI IMAT program is now funding our efforts to optimize DXMS-guided construct design for soluble cancer-implicated proteins (R33 CA099835). Until recently, we thought it unlikely that successful DXMS analysis of membrane proteins would be possible, and this funded grant contains no reference to membrane proteins (IMPs), nor does it support work on them. However, insights and preliminary studies described in the present application now make it likely that, with intensive development work, we can devise highly modified methods that will allow the facile DXMS analysis of IMPs. Development of membrane protein DXMS will greatly impact the structural biology of cancer-implicated IMPs, which are particularly difficult to prepare in crystallizable form. Initial year 1 development efforts will focus on the integrin allbbS, with which I have had considerable experience. Integrins are widely implicated in cancer cell and cancer vasculature biology, and findings with the prototypic allbbS integrin have proven applicable to the understanding of all integrins. The resulting IMP-DXMS methods will be further refined and validated in a year 2 thorough study of additional cancer-relevant IMPs and daughter constructs provided by Dr. Raymond Stevens, PI of the newly NIH-funded JCSG Center for Innovative Membrane Protein Technologies (JCIMPT). Once IMP-DXMS has been fully developed and validated, it will be made available to investigators studying cancer-implicated IMPs by integrating the methods with our soluble-protein DXMS resource now supported by the NCI IMAT program. Thus the NCI's investment in presently funded DXMS work will be greatly leveraged by the relatively modest support requested for the development of IMP-DXMS.

Microfluidic Capillary Assay Utilizing Holographically Encoded Microparticles

Moon, John, CyVera Corporation 1 R43 CA118536-011 (SBIR)

Description: CyVera Corporation proposes to develop and validate the feasibility of a rapid, robust, and inexpensive method for performing multiplexed protein expression measurements. These measurements are needed for the early detection, diagnosis, and management of patients with cancer. This cancer diagnostic platform will be based on the combination of (1) CyVera's newly developed holograpically encoded, multiplexed microparticle assays, (2) self-assembly, and (3) microfluidics. The format we propose will allow rapid and highly sensitive detection of protein expression patterns in small sample volumes and will ultimately lead to a high-throughput instrument platform for cancer diagnostics. In Phase I of this project, prototype microfluidic devices will be constructed with antibody functionalized particles. Batches of individually encoded glass particles will be antibody functionalized, pooled, and self-assembled into microfluidic devices. Once assembled, the identity of each type of particle will be read via its holographic code. Five detection analytes in Phase I will be chosen from a set of putative cancer biomarkers. These commercially available markers will include von Willebrand factor (vWF), C-reactive protein (CRP), albumin, free prostate-specific antigen (fPSA), and complexed PSA (cPSA), all of which have been reported as prostate cancer biomarkers in the literature. The limit of detection and repeatability of each analyte will be assessed via spike-in experiments in serum samples. The goals of Phase I will be to demonstrate (1) <10 pg/mL sensitivity of each multiplexed analyte in a complex sample in under 1 hour, (2) low sample volume requirements of <10 microliters, and (3) ease of fabrication and replication of the microfluidic devices. Success in Phase I will pave the way for the development of an affordable tool for molecular cancer diagnostics and followup patient therapy monitoring.

A New Strategy for Early Detection of Ovarian Cancer

Ratnam, Manohar, Institutional Partner: Innotech Biopharma, Inc., Academic Affiliation: Biochemistry and Cancer Biology, Medical University of Ohio, Toledo 1 R41 CA118540-01 (STTR)

Description: Ovarian cancer is the most common cause of death among cancers of the female genital tract owing to the lack of methods for early detection of the cancer. Despite the identification of a number of serum markers for ovarian cancer, the clinical application of such proteins is confounded by their low tumor expression levels, false-positive tests, and limitations of the assay methods. The glycosyl phosphatidylinositol (GPI)-anchored folate receptor (FR) type A is consistently expressed by nonmucinous adenocarcinomas of the ovary and uterus and in 20 to 30 percent of breast carcinomas. In contrast to the receptor expressed in these tumors, the expression of FR-a in normal tissues is restricted to the luminal surface of certain epithelial cells that is not in contact with the bloodstream. The soluble form of FR-a is a serum marker for the receptor-rich tumors because a large proportion of the membrane-anchored form is shed into the circulation both by proteolysis and by the action of serum phospholipase. Our recent studies strongly support a new methodological concept for the utilization of FR-a that addresses the current limitations of serum markers. Based on mechanistic studies in vitro and tumor xenograft model studies in vivo, we have established that brief treatment with innocuous doses of certain well-tolerated transcription modulators profoundly increases the levels of both membrane associated and serum FR-a produced by FR-a-positive malignant cells but not by the receptor-negative tissues. This finding offers a more effective means for early detection of FR-a-positive tumors. In addition, the ability of FR-a to simultaneously bind both specific antibodies and folate conjugates will allow the development of superior assay methods (increased signal-to-noise ratio) incorporating two levels of selectivity for the protein in the serum. This proposal represents a major step in the development of an innovative technology for the application of FR-a as a serum marker by manipulating its expression and also improving its assay. Upon detection by a serum assay, the tumor may be localized using an available FDA-approved FR-a-targeted imaging agent. In Aim 1, we will further develop antibody and folate conjugate probes to develop simple and high-throughput fluorescent assays for soluble FR-a. In Aim 2, we will utilize malignant cell lines in murine tumor xenograft models to establish optimal treatment regimens for induction of serum FR-a. This initial phase of developing the technology will provide a basis for future clinical studies.

Development of Gene Targeting in *Caenorhabditis Elegans* and *Danio Rerio*

Porteus, Matthew, Hematology-Oncology Division, Department of Pediatrics, University of Texas, Dallas 1 R21 CA120681-01

Description: The goal of this research is to develop methods for the precise modification of specific target genes in two important genetic model organisms, the nematode *Caenorhabditis elegans (C. elegans)* and the zebrafish *Danio rerio (D. rerio)*. Both nematodes and fish are powerful experimental systems that combine elegant developmental biology with large-scale genetics. Both systems have contributed to our understanding of fundamental problems in cancer biology, including programmed cell death, the control of organogenesis, the interaction of cancer susceptibility genes with the environment, and the genetics of melanoma. An important limitation of these model systems is that techniques for site-specific manipulation of the genome are not currently available in either nematodes or fish. Thus, in contrast to murine embryonic stem cells and the yeast *S. cerevisiae*, it is not possible to knock out specific genes or to precisely control the time and place of gene expression. In the last 2 years, a powerful new approach to gene-targeting has been developed and successfully used in flies and in mammalian somatic cells. This technique uses chimeric zinc finger nucleases to stimulate precise targeting of specific genes in their native genomic context. The aim of this proposal is to induce targeted, heritable genetic changes via zinc finger nuclease-mediated homologous recombination in *C. elegans* and *D. rerio*. Initially we will employ a well-characterized zinc finger nuclease that recognizes the green fluorescent protein (GFP) gene. We will introduce the nuclease into transgenic nematodes and zebrafish that express GFP. We expect the resulting double-strand DNA breaks to stimulate mutagenic nonhomologous end joining (NHEJ), leading to the loss of GFP signal. In the second phase, we will simultaneously introduce the nuclease and a repair template that will allow us to create precise mutations in the target locus by homologous recombination. Based on the success of this work, we will then target native genes in the worm and the fish by designing novel nucleases and testing them in vitro and in vivo for activity against the targeted gene. We expect that, if successful, this novel approach

would be a practical, flexible, and powerful technique that would find wide application, significantly increasing the power of these systems to illuminate human cancer biology.

Development of Dynamic Isoelectric Focusing for Cancer Proteomics

Tolley, Luke, Department of Chemistry and Biochemistry, Southern Illinois University 1 R21 CA120691-01

Description: Though great progress has been made in the area of DNA analysis for cancer, understanding the proteins encoded by DNA can provide more answers but is also more challenging. As the study of cancer proteomics advances, it is clear that new analytical tools and technology are needed for the comprehensive profiling of the proteins in a cell so that our understanding of carcinogenesis and the differences between healthy and cancerous cells can progress. Further understanding of cancer proteomics will drive the discovery of new drug targets as molecular changes in the cell are observed without preconceived ideas about what changes would be the most valuable to monitor. Because of the very large number of proteins in a cell, comprehensive analyses require the use of separation methods that have high peak capacities. Capillary isoelectric focusing (cIEF) has shown great promise in this area, with a peak capacity in excess of 1,400. This greatly exceeds traditional separation methods such as liquid chromatography (LC), capillary electrophoresis (CE), or mass spectrometry (MS), which often have peak capacities of less than 200.

An increase in the total peak capacity of a system can be achieved when multiple separation techniques are combined, leading to the popularity and performance of tandem methods such as LC/LC or LC/MS. Though the superior performance of cIEF over CE and LC would seem to make it a preferred choice in a tandem system, it is not able to be efficiently interfaced with other methods. This is the primary reason it is not widely used. The proposed research will continue the development of dynamic isoelectric focusing, which is a new technology developed by the PI that will be able to provide the high peak capacity of cIEF while also efficiently coupling with other techniques. The combined systems made possible will easily outperform other tandem methods and will have a high impact on the molecular analysis of cancer because they will permit the acquisition of a more comprehensive profile of the proteins in cancerous cells than is currently possible. The capabilities of dynamic IEF will be demonstrated by interfacing it to MALDI-MS and using the system to analyze and observe differences in extracts from treated and untreated PC-3 prostate cancer cells. The cell treatment will be based on compounds currently researched by the Co-PI, such as bisdehydrodoisynolic acid, which is an estrogenic carboxylic acid shown to be effective at reducing the proliferation of prostate cancer.

R21 Cancer Vertical Arrays

Welsh, John, Sidney Kimmel Cancer Center 1 R21 CA116214-01A1

Description: One of the experimental challenges in cancer molecular biology is assessing the validity and generality of biomarkers. This has become a critical bottleneck in the development of biomarkers from differential gene expression revealed by microarray studies. In this proposal, we develop the concept of using vertical arrays for exploration of differential gene expression in cancer. Vertical arrays explore the expression of a gene in many biological samples simultaneously, whereas standard microarrays explore the expression of many genes in response to one biological variable at a time. Vertical arrays are like dot blots in this regard, but vertical arrays are printed on glass slides, giving them better signal-to-noise behavior, and rather than spotting the entire complexity of the RNA population in each spot, the RNA population is divided up among multiple spots. These low-complexity representations have superb signal-to-noise performance. The work in this proposal will focus on establishing the feasibility of making a vertical array for studying gene regulation in many cancer samples simultaneously. Potential throughput is very high, such that multiple regions from each tumor can be studied simultaneously. This approach will be useful in confirming that a gene is indeed differentially regulated, in determining the distribution of expression of the gene in the transformed and surrounding normal tissue, and in determining whether the gene behaves in a similar manner in different cases of the same type of cancer and in different kinds of cancer. The goals require extensive and efficient microdissection, and we have built a novel instrument, the "tissue mill," to achieve these ends. Relevance: Biomarkers are useful for diagnosis and prognosis and as potential therapeutic targets for cancer. There are hundreds of potential biomarkers, but further validation is needed before they can be exploited.

High-Resolution Analysis of Linear Genomic DNA in Parallel Nanochannel Arrays

Cao, Han, Bionanomatrix 1 R43 CA120611-01 (SBIR)

Description: We are developing a nanochip device for manipulating long genomic DNA for high-resolution (kilobase), whole-genome analysis of cancer biomarkers such as gene amplifications, deletions, and translocations. These chromosome structural aberrations are strongly implicated in the process of malignant transformation and are important diagnostic, prognostic, and therapeutic indicators for many types of cancer. Although PCR offers the ultimate (single-base) resolution for detecting and analyzing these anomalies, it is impractical for scanning the entire genome in a comprehensive, linear fashion. Techniques that rely on probing chromosomes, such as metaphase FISH, while providing a pangenomic view, cannot resolve structures below the Mb range. By probing uncompressed interphase DNA, resolution can be improved, but spatial organization of the genome is lost, so multiplexed and quantitative information is difficult to obtain. By stretching out (linearizing) interphase DNA, using techniques such as "molecular combing" or "optical mapping," it is possible to probe specific loci in a spatially significant way, with resolutions in the kb range. However, techniques for mechanically linearizing DNA are inherently variable, leading to inconsistent stretching of molecules, which often cross over and retract upon themselves. This makes it difficult to standardize such techniques as high-throughput methods for the biomedical community. We are developing an innovative alternative to mechanical stretching of DNA. We have found that individual DNA molecules, because of the self-avoiding nature of the DNA polymer, will elongate and straighten in a consistent manner when streamed into confining nanometer-scale channels (nanochannels). We have used a novel nanoimprint lithography technique to reliably manufacture nanochannel structures in silicon chips and have demonstrated that DNA in these nanochannels can be visualized and their dimensions measured. We now ask the question, can we quantitatively interrogate this linearized DNA with locus-specific probes for the detection of chromosome structural aberrations associated with cancer? Our product, the nanochannel array chip, will be part of an integrated platform for the routine and standardized quantitative analysis of DNA structure that will enable archiving and cross-laboratory comparison of data.

A Novel Diagnostic Assay for Oncogenic Human Papillomaviruses

Lu, Peter, Arbor Vita Corporation 2 R44 CA121155-01 (SBIR)

Description: Arbor Vita Corporation (AVC) has developed a novel cervical cancer diagnostic based on an understanding of the biology of human papillomavirus (HPV). HPV infection is one of the most common sexually transmitted diseases, with an estimated 5.5 million new infections per year in the United States alone. High-risk (oncogenic) HPV types are correlated with virtually all cervical cancers. Pap smear and liquid-based cytology screenings have greatly reduced the incidence of cervical cancer, but the Pap test has both high false-negative and false-positive rates and requires an extensive infrastructure of trained cytologists to interpret the results. A cheaper test with greater reliability and predictive value would be of great clinical benefit. The virally encoded E6 and E7 proteins of high-risk HPV types have been shown to be essential for cell transformation and cancer progression, and E6 proteins from high-risk HPV types, but not low-risk HPV types, are known to bind cellular PDZ domains. AVC has extended those studies and demonstrated a perfect correlation between high-risk HPV and E6-PDZ binding. Based on these findings, we have developed a novel cervical cancer diagnostic assay of HPV E6 using PDZ protein capture. In our SBIR Phase I, AVC developed a novel PDZ capture sandwich ELISA test for HPV E6 that detects over 75 percent of high-risk HPV types and demonstrated its utility with human cancer samples. We were able to begin quantifying E6 from cells and improved sensitivity to allow E6 detection in a much smaller sample than typically collected in a Pap test. In Phase II, we propose to complete development of our prototype PDZ-based cervical cancer test in preparation for clinical trials. Specifically, we propose to (1) expand our antibody detection to include 95 percent of known high-risk HPV types, (2) optimize clinical sample handling for E6 protein detection, (3) optimize the PDZ/antibody sandwich ELISA for clinical laboratory implementation, and (4) extend the studies correlating high-risk E6 protein levels and clinical cytology staging.

Enhanced Cancer Microarray

Szmacinski, Henry K., Microcosm, Inc. 2 R44 CA118466-01 (SBIR)

Description: The fight against cancer, heart disease, HIV infection, and all diseases is slow. Lives are being lost. The public spends over $1 trillion per year on health care. The government spends that much again. The tool used most in the fight against cancer employs fluorescent dyes to indicate the presence of cancer, to discover new ways to test for cancer, and to discover medicinal cures. Millions of tests are performed each year using thousands of tiny spots on a microscope slide, called a microarray. These spots fluoresce when a sought-after biological indication is present. The problem is that this ubiquitous method of research and diagnosis can detect less than half of the biological information needed to end cancer because the fluorescent light signal is very weak. The proposed project will increase the sensitivity of fluorescence assays 1,000-fold. This innovation will revolutionize the battle against cancer and all diseases. The mission of the NCI is to discover and develop new technology for the fight against cancer. This project will continue the development of metal-enhanced fluorescence (MEF) begun under a previous Phase I SBIR grant from the NCI. MEF uses thin-film technology to deposit layers of metal particles and dielectrics on microarray substrates. MEF has been repeatedly proven to increase the fluorescence assay sensitivity 40- to 100-fold. This project will transfer MEF technology to a manufacturing environment, optimize the manufacturing protocols, and pilot-test the first products. Cancer DNA assays will be performed at the NCI Microarray Center to evaluate and validate the performance of the new product. In addition, this project will integrate two commercially available assay technologies with the MEF microarray. The active surface of a microarray substrate must be able to bind biological material efficiently. GenTel BioSurfaces, Inc., provides surface chemistry that is proven to increase binding by a factor of 7-10 compared to all other available substrates. Their surface chemistry combines with MEF to produce an assay sensitivity increase in the range of 100 to 1,000. Martek sells super bright fluors that are proven to increase the fluorescence signal 20- to 300-fold compared to conventional fluors. Microcosm has proven that MEF increases the light output of Martek fluors another 40-fold. The integration of Martek labeled assay reagents, Gentel's surface chemistry, and Microcosm MEF substrates promises a multiplicative increase in assay sensitivity exceeding 1,000-fold. Martek and Gentel products are on the market. Based on over 3 years of development at Microcosm, this integrated MEF substrate is ready for commercialization. With this project, a universally needed and revolutionary new microarray assay product line can enter the market in less than 2 years.

Droplet Cell Array Assays

Faris, Gregory, SRI International 1 R21 CA118526-01A1

Description: Understanding the basic genetic and molecular markers of cancer at the cellular level is vital for preventing, diagnosing, and treating cancer. Recent work in our laboratory has led to new technologies for manipulating small aqueous drops containing biological molecules. We use laser light to induce surface tension gradients, which allows us to selectively move very small individual droplets (nL-pL), and we have performed simple enzymatic assays with this approach. Because our technique uses laser heating as a basis for droplet control, we believe that it is well suited to genomic analysis methods, such as polymerase chain reaction (PCR), that rely on thermal cycling. We propose to combine our laser-based droplet control techniques with genomic analysis tools to develop a device for screening large numbers of individual cells. To test the capabilities of our apparatus, we propose an R21 project with the following Specific Aims: (1) Optimize and automate the liquid handling system for genomic analysis. We will choose the optimal materials and reagents for use in our apparatus. We will automate both the droplet delivery and the droplet handling capabilities of our device. (2) Perform real-time PCR in our droplet-based system. We will test the overall sensitivity and quantify nonspecific amplification in our binary liquid system. (3) Examine single cells within small droplets. This examination will include testing for short- and long-term cell viability and PCR amplification of single-cell genomic material. This research is fully consistent with the goals outlined in RFA CA06002. If this program is successful, we believe that the resulting technology will prove invaluable in helping physicians and scientists better understand the molecular basis of cancer while also providing tools to help diagnose specific variants of disease, plan and assess therapy, and monitor disease recurrence.

Direct Detection of Hypermethylation in Cancer

Ghosh, Indraneel, Department of Chemistry, University of Arizona 1 R21 CA122630-01

Description: The long-term objective of the proposed research project is to provide a robust, sensitive, and rapid method for the direct detection of CpG island methylation in the promoter region of specific genes implicated in cancer. Cytosine methylation occurs at CpG dinucleotides in 70 to 80 percent of the human genome, most often in repetitive genomic regions. On the other hand, CpG islands, defined as short sequences with statistically high CpG content and present in the promoter region of many genes (60 percent), are primarily protected from methylation in normal tissues. These CpG islands have been found to be methylated in cancer leading to transcriptional repression. Recent experiments provide strong correlation between CpG hypermethylation at promoter sites of numerous genes and the incidence of cancer, thus making specific promoter hypermethylation a valuable marker for early detection. Current methods for detection of specific CpG island methylation rely on extensive bisulfite treatment of methylated DNA followed by PCR-based amplification, sequencing, or microarray techniques. These current methods, though powerful, are also laborious, time-intensive, and expensive for characterizing known sites of hypermethylation. Toward the goal of rapidly determining promoter CpG hypermethylation, we will apply our newly developed technology called Sequence Enabled Reassembly (SEER) of proteins. The SEER system allows for the recognition of specific sequences of double-stranded DNA that result in the concomitant assembly of functional protein reporters (green fluorescent protein and p-Lactamase). In the proposed detection of specific CpG hypermethylation, we will target CpG islands utilizing the methyl-CpG binding domain (MBD) of the MBD2 protein, while targeting the correct promoter sequence utilizing a designed zinc-finger. Our approach has the potential to provide a sensitive turn-on sensor for directly reporting upon CpG methylation at known promoter sites. This approach if successful will rapidly distinguish between normal and cancerous tissues in a clinical setting without the requirement for bisulfite treatment, PCR amplification, and sequencing. We will provide proof of concept by (1) designing and optimizing turn-on biosensors for detecting specific methylation events in model DNA constructs and (2) designing and testing biosensors that target promoter regions of genes (BRCA1, CDH1, p15, p16, MGMT, GSTpI) implicated in cancer.

Novel Image-Based Screening of Mammary Tumors

Knowles, D., Livermore Laboratories, University of California, Berkeley 1 R33 CA1184791-01A1

Description: Though great progress has been made in the area of DNA analysis for cancer, understanding the proteins encoded by DNA can provide more answers but is also more challenging. As the study of cancer proteomics advances, it is clear that new analytical tools and technology are needed for the comprehensive profiling of the proteins in a cell so that our understanding of carcinogenesis and the differences between healthy and cancerous cells can progress. Further understanding of cancer proteomics will drive the discovery of new drug targets as molecular changes in the cell are observed without preconceived ideas about what changes would be the most valuable to monitor. Because of the very large number of proteins in a cell, comprehensive analyses require the use of separation methods that have high peak capacities. Capillary isoelectric focusing (cIEF) has shown great promise in this area, with a peak capacity in excess of 1,400. This greatly exceeds traditional separation methods such as liquid chromatography (LC), capillary electrophoresis (CE), or mass spectrometry (MS), which often have peak capacities of less than 200.

An increase in the total peak capacity of a system can be achieved when multiple separation techniques are combined, leading to the popularity and performance of tandem methods such as LC/LC or LC/MS. Though the superior performance of cIEF over CE and LC would seem to make it a preferred choice in a tandem system, it is not able to be efficiently interfaced with other methods. This is the primary reason it is not widely used. The proposed research will continue the development of dynamic isoelectric focusing, which is a new technology developed by the PI that will be able to provide the high peak capacity of cIEF while also efficiently coupling with other techniques. The combined systems made possible will easily outperform other tandem methods and will have a high impact on the molecular analysis of cancer because they will permit the acquisition of a more comprehensive profile of the proteins in cancerous cells than is currently possible. The capabilities of

dynamic IEF will be demonstrated by interfacing it to MALDI-MS and using the system to analyze and observe differences in extracts from treated and untreated PC-3 prostate cancer cells. The cell treatment will be based on compounds currently researched by the Co-PI, such as bisdehydrodoisynolic acid, which is an estrogenic carboxylic acid shown to be effective at reducing the proliferation of prostate cancer.

Novel Probe for Quantitative Imaging of Gene Expression

Tsourkas, Andrew, Department of Bioengineering, University of Pennsylvania 1 R21 CA116102-01A1

Description: We propose to develop a molecular imaging probe that will provide quantitative information on the expression level of mRNA with spatial and temporal resolution. Specifically, an oligonucleotide-based probe will be designed to form a stem-loop structure and will be labeled with a "reporter" fluorophore at one end and a quencher at the other, analogous to a molecular beacon; however, the oligonucleotide will also be labeled with a second optically distinct "reference" dye/nanoparticle, which will be selected such that it is unquenched regardless of the conformation of the probe. Fluorescently labeled neutravidin and quantum dots will be tested for their suitability in serving as the reference dye. We hypothesize that beneficial features of this novel probe compared with conventional molecular beacons will include (1) the ability to monitor transfection efficiency due to the presence of the unquenched reference dye, which will reduce false negatives by allowing for the differentiation between untransfected cells and cells with low levels of gene expression; (2) the ability to remove via ratiometric imaging (i.e., reporter fluorescence/reference fluorescence) the impact of instrumental and experimental variability; (3) the ability to quantitatively compare variations in gene expression levels between samples, between cells within individual samples, and even between subcellular compartments by using the reference dye as a point of reference; (4) the ability to quantify gene expression with spatial and temporal resolution since the covalent linkage between the reporter and reference dye ensures that they exhibit an equivalent intracellular lifetime and colocalization pattern; (5) the ability to use the quantum dot/neutravidin as a platform to attach targeting agents, opening up the possibility for in vivo imaging; and (6) the possibility of an improved signal-to-background due to quenching of the "reporter" dye by both the quencher molecule and the "reference" dye. To evaluate these features, we will pursue two major aims during the proposed research: (1) design, synthesize, and characterize the "quantitative" molecular beacon (QMB) in terms of its signal-to-background and lower detection limit (in vitro and in vivo) and (2) evaluate the ability of the QMBs to quantify endogenous mRNA expression in breast cancer cells in real time. It is envisioned that the approach proposed here will allow significant advancements in our understanding of human health and disease and could potentially prove to be a powerful diagnostic tool.

Fluorescent Activity Sensors in Analysis of Oncogenic P13K/Akt Signaling

Zhang, Jianxuan, Pathology Department, Weill Medical College of Cornell University 1 R21 CA122673-01

Description: Activation of phosphatidylinositol 3-kinase (PI3K) and the downstream serine/threonine kinase Akt (also known as protein kinase B) triggers a cascade of responses that are critical for tumorigenesis, from cell growth and proliferation to survival and mobility. Aberrations of components in the PI3K/Akt pathway have been shown to be present in a majority of tumors. We hypothesize that aberrant PISK/Akt activation could be characterized by combined activity profiles and used as a diagnostic marker in cellular activity profiling. To test this hypothesis, we propose the following Specific Aims: (1) analyze the activities of PI3K and Akt in breast cancer cell lines and to further develop fluorescent activity sensors for various components in the PISK/Akt pathway and (2) develop cellular assay platforms for high-throughput activity profiling of oncogenic PISK/Akt signaling. These studies will take advantage of a series of fluorescence resonance energy transfer (FRET)-based reporters we have recently developed for measuring the activities of Akt and PI3K in living mammalian cells. Fluorescent activity sensors and cellular assay platforms developed in this study can be used in the systematic analysis of the critical components in PISK/Akt pathway in various cancers to generate activity profiles. Correlation of genetic alterations with activity profiles and phenotypes should provide new insights into the molecular mechanisms of cancer development. On the other hand, molecular diagnostics based on such activity profiling could identify the molecular defects and the malfunctioned key nodes in the signaling network for a given cancer and guide appropriate molecular therapeutics as well as facilitate their development and evaluation.

Genotyping Arrays as a Prognostic Tool: Glioma Model

Chernova, Olga, Institutional Partner: Cleveland Biolabs, Inc. Academic Affiliation: Department of Clinical Pathology, Cleveland Clinic Foundation 1 R41 CA110584-01A2 (STTR)

Description: The development of cancer patient pretreatment screenings that utilize genetic markers to stratify the patient to a particular therapy is one of the main new directions in improving the quality of anticancer therapies. Gliomas, the most common primary brain tumors, represent a specific type of cancer with well-characterized prognostic genetic markers that predict patient survival and response to chemotherapy. These alterations include loss of chromosomes 1p, 19q, and 10q; gain of chromosome 7 and 19q; amplification of oncogenes; and deletions/mutations of tumor suppressor genes PTEN, TP53, and CDKN2A. Routine genotyping of glioma patients for loss of 1 p/19q and amplification of the epidermal growth factor receptor (EGFR) gene has been available at the Cleveland Clinic since 2001 and has already affected the treatment of oligodendroglioma patients. Still, only a few molecular markers are routinely tested in a number of leading clinics in the United States. Current genotyping assays are based on loss of heterozygosity (LOH), fluorescent in situ hybridization (FISH), and direct sequencing techniques, which are time consuming and focused on only a handful of molecular markers. We propose the development of a high-throughput genotyping assay that will provide simultaneous analysis of multiple genetic alterations, including mutations of selected genes, allelic imbalances, and copy number alterations. The assay will be focused on previously identified molecular prognostic markers, including both well-validated, as well as recently discovered, markers with potential prognostic value. This combination will accelerate validation of novel markers through fast accumulation of data on genetic alteration in tumor specimens. The assay technology is based on custom oligonucleotide microarrays manufactured by NimbleGen Systems, Inc., using recently developed maskless lithography, which allows easy adjustment and replacement of the oligonucleotide probes as necessary. The sample preparation procedures will be adjusted for use of tumor DNA derived from formalin-fixed paraffin-embedded tissue from the pathology archive. Analysis of archival tissues will enable us to perform retrospective studies using clinically and genetically characterized tumor specimens. The significant increase in the number of analyzed genetic markers will result in more accurate prognosis at lower cost, will lead to the discovery and validation of additional prognostic markers, and will improve treatment stratification of glioma patients.

Measuring c-Abl Activity in CML Using a High-Affinity Nanosensor

Gellibolian, Robert, SynBiogen, Inc. 1 R43 CA120937-01 (SBIR)

Description: The ability to monitor and measure protein kinase activity in tumorigenesis and cancer can be indicative of and critical to the transformation process and therefore represents an attractive diagnostic strategy, with a multibillion dollar market opportunity. In this proposal, we aim to develop a novel, high-affinity fluorescent nanosensor and homogeneous assay system for monitoring the phosphorylation of a bona fide peptide substrate by c-Abl, an important kinase involved in the etiology of chronic myelogenous leukemia (CML), using FRET. The technology deployed during this phase of the proposal will directly translate into the development of a sensitive platform for the diagnosis of CML. In Specific Aim 1, we will synthesize the high-affinity nanosensor. In Specific Aim 2, we will develop a sensitive one-step assay using this reagent. Specific Aim 3 will elaborate on this and test the effectiveness of the nanosensor in measuring c-Abl activity in CML-positive cell lysates. Given the fact that ~400 disorders such as cancer have been associated with protein kinases, the development of a family of sensitive nanosensors such as the one proposed in this grant application will facilitate the diagnosis of these diseases sensitively and selectively, therefore becoming of paramount importance, and will find immense utility in all the various facets of our health care, from drug discovery to patient health and point-of-care diagnostics.

FY 2007

Development of DNA-Templated Infrared Quantum Dots

Kelley, Shana, Department of Biochemistry, University of Toronto 1 R21 CA122878-01A2

Description: Functionalized semiconductor quantum dots have previously been demonstrated to bind to markers on cells. Through their spectrally narrow optical emissions, they illuminate tumors and other harbingers of disease. They are enabling the highly specific detection of a range of diseases at the earliest stages. We propose to pursue a new and improved class of semiconductor quantum dots. We have already shown that, by seeding the growth of nanoparticles using a DNA template, we are able to produce quantum dots that are efficient, stable emitters in the infrared wavelengths—a so-called "biological window" in which living organisms are much more transparent than in the visible wavelengths, and in which living organisms' autofluorescence is orders of magnitude lower than in the visible. These materials retain their luminescence properties over time even in biological media such as plasma at 37 °C. The collaborating team of Dr. Shana Kelley, a nucleic acids chemist, and Dr. Edward Sargent, an optoelectronics engineer, bring the expertise required to optimize the DNA-grown nanoparticles for applications in medical diagnosis. The strategy of directing the growth of luminescent nanoparticles using DNA provides a one-pot route toward the strong coupling of light-emitting tags with DNA-based aptamers for programmable specific binding. The project is divided into the following specific aims: (1) Direct the growth and thereby maximize the performance of DNA-templated quantum dots using designer DNA sequences and (2) discover optimized DNA sequences for specific binding assays using in vitro selection. We will thereby develop a new means of creating highly luminescent nanomaterials for medicine and biology. The team, with its complementary expertise in biomolecular chemistry and optical materials, is equipped with the infrastructure and resources to make a significant contribution to the realization of improved visible and infrared fluorophores for diagnosis.

Diagnostic Analyses of Endogenous Protein Interactions

Landegren, Ulf Dag, Uppsala University 1 R21 CA126727-01A1

Description: Detection of patterns of interactions among proteins can prove of great conceptual and diagnostic value in malignancy and other diseases, but efficient methods have been lacking to characterize interactions in both research and diagnostics. We will take advantage of a unique set of tools developed in our laboratory to establish methods to (1) characterize interactions among large sets of proteins directly in patient samples, using the proximity ligation technique and a recently established paired-tag array readout, for interaction biomarker discovery and (2) develop and validate smaller scale diagnostic assays of interacting protein molecules using a combination of array readout and a novel in situ interaction analysis that we have also recently established. The procedure we will use in the characterization phase involves immobilization of in situ interacting proteins cross-linked to a solid support. A series of antibodies specific for proteins of interest, conjugated with oligonucleotides including antigen-specific tag sequences, are allowed to bind to proteins in the biological samples in so-called proximity ligation reactions. The presence of interacting proteins results in co-localization of specific pairs of antibodies, which in turn brings their attached oligonucleotides into close proximity, allowing them to be joined by enzymatic ligation. The resulting DNA molecules are combinations of tags that reflect the identity of the detected pairs of interacting proteins. The ligated molecules are amplified, restriction digested, and sorted on an array containing all possible pairs of tag sequences for the proteins under investigation. Paired-tag sequences properly hybridized to the arrayed molecules can be circularized, and the oligonucleotides immobilized on the array next serve as primers for a rolling-circle reaction in which only correctly ligated, circular molecules are amplified. The resulting rolling-circle products contain tandem repeats of a generic detection sequence, permitting hybridization of a fluorescent probe for array detection. The method will be established and applied to study interactions among members of the Smad protein family, intracellular effectors of the transforming growth factor alpha (TGF-α) signaling pathway that plays well-documented roles in both tumor suppression and tumor progression. In the second phase, which will temporally overlap with the first, assays adapted for diagnostic use will be developed, and interacting pairs of proteins will be validated as markers using this assay, and also by studies in tissue sections from patient samples, with the help of so-called proximity ligation in situ assays.

In these assays, proximal binding of pairs of antibodies with attached oligonucleotides result in the formation of circularized DNA strands for localized rolling-circle replication reactions that permit even single interacting pairs of proteins to give rise to clearly detectable signals. The proposed procedures allow endogenous protein complexes to be observed without the requirement for cloning or transfecting exogenous components, permitting dynamic interaction networks to be investigated in normal, precancerous, and cancerous cells. Furthermore, any pairwise interactions among a given set of proteins can be monitored, and the paired-tag system is scalable and transferable to study different pathways. The procedures and results of this project will establish protein interaction events as markers for tumor diagnostics and for evaluation of drug treatment.

Microfluidic MALDI-MS Device for High-Throughput Proteomics and Biomarker Discovery

Lazar, Maria Iuliana, Bioinformatics Institute, Virginia Polytechnic Institute and State University
1 R21 CA126669-01A1

Description: Cancer is a leading cause of death in the United States, with more than 1,000,000 new cases diagnosed every year. As a result of the high sensitivities and specificities that are required to justify large-scale population screening, only very few single protein biomarkers are routinely used today in the clinical setting. It is of critical priority to develop novel technologies that will enable the rapid detection of a plethora of biomarkers relevant to early diagnosis, prognosis, staging, and treatment response. The long-term objective of this research is to combine the emerging technology of microfluidics with state-of-the-art mass spectrometry (MS) detection to enhance our capacity for analyzing molecular structure and function in biological systems. This application capitalizes on the distinguishing capabilities of microfluidic architectures that enable process integration, multiplexing, fast and high-throughput processing of minute amounts of sample, and the power of MS detection that provides the sensitivity, specificity, and resolving power necessary for unambiguous detection of trace level components. Specific Aim 1 of this project is to develop a compact, low-cost, and disposable microfluidic analysis platform with matrix-assisted laser desorption ionization (MALDI)-MS/MS detection for high-throughput proteomics that will enable the study of protein co-expression patterns and biomarker discovery. The microfluidic device will carry out parallel liquid chromatography (LC) separations and will integrate a novel microchip-MS interface to enable sensitive MALDI-MS/MS detection directly from the chip. Specific Aim 2 of the project is to demonstrate the effectiveness of the microfluidic MALDI-MS/MS platform for the detection of multiple cancer biomarkers in cellular extracts. Cellular fractions from the MCF7 breast cancer cell line will be analyzed for target proteins that are involved in essential cellular processes driving cancer onset and development (cell proliferation, cell cycle regulation, DNA repair, apoptosis, and invasion/metastasis).

Microfabrication Tools for In Vitro Monitoring of Cancer Cell Metabolism

Revzin, Alexander, Department of Biomedical Engineering, University of California, Davis 1 R21 CA126716-01A1

Description: The physiological or pathophysiological state of cells and organs is reflected in their energy metabolism. For example, deviations in glucose metabolism are almost always observed in tumors. The energy metabolism of cancer cells differs strikingly from normal tissue; glycolysis and subsequent lactate production are much more prominent in cancer cells, even under aerobic conditions. Increasingly, therapeutic strategies for cancer treatment are designed to target metabolic deviations, making levels of energy metabolites such as glucose or lactate potential correlates of drug efficacy. The goal of this proposal is to develop a lab-on-chip platform for in vitro monitoring of the effects of drug candidates on energy metabolism of cancer cells. The proposed platform will intimately integrate small groups of glioma (brain cancer) cells with miniature glucose biosensors in a microfabricated device, where the cellular microenvironment can be precisely defined and easily modulated. This platform will be used to (1) establish tumor-mimicking microenvironment conditions (e.g., hypoxic, acidic, nutrient-limiting) in glioma cell cultures, (2) challenge the cells with pharmacological inhibitors of kinase signal transduction pathways central in energy metabolism, and (3) monitor local extracellular glucose levels. Thus, the cell culture/biosensor platform will connect tumor microenvironment, energy metabolism, and anticancer drug efficacy and will help to delineate conditions, making cancer cells more susceptible to therapy. The proposed technology will be a valuable tool for the development of therapeutic anticancer agents and will help illuminate molecular aspects of metabolic adaptation of cancer cells.

Proteomic Profiling of Cancer-Related Redox Signaling Pathways

Poole, Leslie, School of Medicine, Department of Biochemistry, Wake Forest University Health Sciences
1 R33 CA126659-01A1

Description: It is widely appreciated that reactive oxygen species (ROS) play a major role in the initiation of cancer, and they are also implicated in many cancer therapies, such as ionizing radiation, cisplatin, and taxanes. More recently, it has been discovered that cancer cells produce ROS as signaling molecules that promote proliferation. Unfortunately, the molecular details of how redox regulation affects cell signaling events are far from clear. New experimental and computational technologies that we have developed are uniquely suited to identifying the molecular targets that are modified by ROS, as a result of either ROS damage or ROS signaling. With the reagents and methods that we have recently developed, we can now evaluate the "redox profile" of cell populations by targeting uniquely reactive cysteine sulfenic acid (Cys-SOH) groups, the initial intermediates generated following reaction of activated protein thiolate groups with hydrogen peroxide and peroxynitrite (and perhaps other ROS). In this R33 application, our labeling technology will be further developed for quantification and multiplex analysis to have broad applicability in (1) investigation of basic mechanisms of ROS damage and ROS signaling, (2) molecular profiling to stratify patients with cancers that are sensitive to ROS-generating therapies, and (3) development of novel cancer therapies based on the inhibition of ROS-dependent proliferative signaling. The following specific aims are proposed: (1) to develop reagents and methods of use for additional new, multicolor fluorescently labeled Cys-SOH reagents for multiplex analysis of samples; (2) to develop quantitative mass spectrometry methods, which have some major advantages over gel-based methods (including direct readout of protein identity and numerous posttranslational modifications); and (3) to use the new quantitative methods to detect and identify Cys-SOH modified proteins generated during ROS-dependent signaling in HEK-293 cells and ovarian cancer cells. Taken together, the approaches developed in Specific Aims 1 and 2 will provide new tools for the research community to study the mechanisms of redox regulation and signaling. In Specific Aim 3, these tools will be used to determine the targets of ROS in the regulation of cell proliferation and apoptosis. First, we will continue our study of NF-κB regulation in HEK-293 cells in response to cytokine (TNF-α) and tumor promoter (TPA) stimulation. Second, we will use ovarian cancer cells treated with cisplatin or taxane to determine which protein oxidations are critical to regulating survival and apoptosis. Besides providing specific information about the mechanism of redox regulation and signaling, these biological experiments will allow us to further refine our reagents and methods to make them most useful to the cancer biology community. These approaches to detecting functional oxidative modifications to cellular proteins hold promise in identifying specific protein targets that mediate the actions of anticancer drugs, e.g., through their effects on cell cycle arrest, cell division, or apoptosis. An outgrowth of these studies could also be the development of new anticancer drugs and the ability to predict the efficacy of a given drug in the treatment of individual patients.

Quantitative, Multiplexed, and High-Throughput: Macroarrays of Lysate Microarrays

Macbeath, Gavin, Department of Chemistry and Chemical Bio, Harvard University 1 R21 CA126720-01A1

Description: Mammalian signaling networks comprise biochemical pathways with shared components, common inputs, and overlapping outputs. Understanding how information flows through these pathways requires information on signaling networks as a whole, rather than on one or two components. To study signaling at a systems level, we need ways to measure the abundance and posttranslational modification of many proteins in a parallel, quantitative, and reliable manner. In addition, since an understanding of signaling requires the frequent temporal sampling of many proteins under multiple conditions, these methods must be high-throughput. Here, we describe technology that mimics an immunoblot, but in a multiplexed and extremely miniaturized format. Cells are cultured in 96-well plates and subjected to a variety of perturbations (stimulation with epidermal growth factor in the presence of selected shRNAs or cDNAs). The cells are then lysed and the lysates arrayed at high spatial density onto glass-supported nitrocellulose pads, also arranged in a 96-well format. By probing each pad with a different antibody, the "state" of the signaling network is assessed. Currently, high-throughput multidimensional readouts can be obtained either by automated fluorescence microscopy or by multiplexed flow cytometry. Although both techniques provide the ability to

track more than one protein simultaneously, they rely on the use of different colored fluorophores and hence can only follow about a dozen proteins. In contrast, the technology described here enables a single sample to be replicated thousands of times on separate microarrays and is thus easily scaled. This application details efforts to make lysate microarray technology rigorously quantitative and outlines automation strategies that render it high-throughput and reproducible. In addition, since one of the biggest challenges in analyzing cancer at a systems level is to go beyond a mere description of the data, a strategy is also presented to build predictive models of cell signaling using Bayesian methods. As proof of concept, we will focus on epidermal growth factor signaling in A431 cells. Although this system is relatively well understood, our approach should capture higher order interdependencies between proteins that are not evident from traditional studies. More importantly, our strategy should provide a general way to uncover causal relationships in less well-studied networks using data derived from our high-throughput microarrays.

High-Performance Affinity Reagents for Peptide Epitopes

Koide, Shohei, Department of Biochemistry and Molecular Biology, University of Chicago 1 R21 CA132700-01

Description: Cancer results from the disregulation of signal transduction networks. Thus, quantitative detection, functional assessment, and isolation of proteins involved in cancer biology are critically important for molecular analysis of cancer. Short peptide motifs within, and posttranslational modification of, many members of this class of proteins play central roles in signal transduction regulation through controlling protein function and interactions. However, there are a very small number of affinity reagents (reagents that bind to a target with high affinity and specificity) to this class of high-value epitopes in cancer analysis. The long-term goal of this project is to develop a powerful technology platform for generating high-performance affinity reagents for short peptide epitopes. The primary products will be novel affinity reagents termed "epitope clamps." Our strategy is distinct from many others in that we aim to develop distinct types of affinity reagents, with each type specific to a particular class of short peptide epitopes. This contrasts with the conventional antibody-based approaches in which a single, general-purpose platform is used for engineering distinct types of targets. Our innovative protein engineering strategy harnesses the inherent specificity present in the so-called interaction domains and dramatically enhances their affinity and specificity by attaching an "enhancer domain." This makes it possible to generate protein libraries predisposed to binding to a particular motif, thereby dramatically increasing the chances of successfully engineering high-performance affinity reagents. Our proof-of-concept experiments have successfully demonstrated this new paradigm in affinity reagent engineering. These proposed studies will establish the full potential of the epitope clamp technology and will generate high-performance affinity reagents to phosphopeptide epitopes in proteins that are critically involved in cancer biology. The technology and tools developed in this project will make a major impact on molecular analysis of cancer and, more broadly, on proteomics and biotechnology. Accurately measuring the amounts of proteins of interest in cells and tissues and assessing their functional state are major technological challenges in molecular analysis of cancer. This application aims to establish a powerful technology platform for the facile generation of high-performance reagents that tightly bind to a predefined segment within a protein of interest. We aim to develop such "affinity reagents" to diverse protein segments that carry a chemical signature of activated proteins. This technology will fill a major void in the current technology portfolio for the molecular analysis of cancer.

Standardized NanoArray PCR for Gene Expression Profiling of Lung Cancer

Morrison, Tom, Medical University of Ohio, Departments of Pathology, Medicine, Public Health, and Biology, Biotrove, Inc. 1 R21 CA132806-01

Description: Molecular characterization of cancer, in particular by gene transcription profiling, has great potential to improve prognosis, therapeutic selection, and clinical outcomes. However, the potential for using expression signatures for cancer prognosis and treatment selection is hampered by the lack of readily deployable test kits with the accuracy, low RNA requirement, and intersite concordance required for routine clinical use. We propose an innovative solution based on two well-validated polymerase chain reaction (PCR) technologies whose combination uniquely addresses the problem of diagnostic assay reproducibility. Our plan is to implement Standardized RT (StaRT)-PCR, a proven competitive PCR method developed at the University of Toledo, in a novel nanofluidic PCR platform developed by BioTrove, Inc., in order to streamline the fluidic workflow, improve measurement throughput, and at the same time reduce test cost and maintain low RNA input. As compared with existing hybridization or real-time quantitative PCR (qPCR) approaches, Standardized NanoArray PCR (SNAP) will provide the same dynamic range and quality as real-time (RT)-PCR yet require less RNA input and be more readily clinically deployable. The development will entail stepwise integration of proven technologies. First, RT-qPCR TaqMan assays will be developed for 16 lung tumor prognostic genes. These assays will be converted to StaRT-PCR by the creation of a competitive template and a competitor-specific, dye-labeled probe. Adding a pre-amplification step to StaRT-PCR will reduce the RNA input requirements to enable thousands of tests per sample. Finally, moving the assays into the OpenArray nano-PCR plate will streamline fluid handling. By using RNA isolated from lung clinical tumor resections, dynamic range and precision equivalent to RT-qPCR will be demonstrated for the integrated platform. After the initial development phase is complete, we will compare SNAP and RT-qPCR in two critical gene expression profiling experiments. First, we will compare the minimum amount of RNA required for each method by monitoring loss of precision as a function of decreasing RNA sample input. Second, we will demonstrate lower intersite variability, a critical factor for deployability, by measuring the gene expression profiles of seven lung tumor resection samples in three laboratories. Meeting these specific aims will lead to seeking further funding for multisite prognostic validation studies involving formalin-fixed, paraffin-embedded (FFPE) lung specimens with extensive clinical history.

Novel Methods to Localize Protein-Protein Interactions in Fixed Cells and Tissues

Camp, Robert, Department of Pathology, Yale University 1 R21 CA125277-01A2

Description: Although analyses of DNA, RNA, and protein expression can elucidate the phenotype of a cell, ultimately it is the interactions between proteins that determine metabolic function. Understanding protein-protein interactions is vital to the study of cancer, and the aim of most new chemotherapeutic drugs is to disrupt aberrant interactions. Recent advances in the quantification of tumor biomarkers in patient samples show great promise in predicting patient outcome and response to treatment. However, there is no good way to assess the co-localization of proteins in tissue samples. The successful evolution of biospecific therapies and associated pharmacodiagnostics may ultimately require quantitative measures of protein-protein interactions within each patient's tumor. From the basic science perspective, new methods in co-localizing proteins in tumor samples will advance the understanding of carcinogenesis and the subsequent development of targeted therapies. We hypothesize that engineering robust methods for assessing protein-protein interactions in human tumor samples will significantly improve the analysis of patient specimens and ultimately speed the development of targeted patient-specific therapies. Protein interactions are the gears that drive cells. In tumor cells, many of these processes are corrupted. Today, we can study the expression of individual proteins in patient tumor samples; however, we cannot look at the interactions between these proteins in patient tumors. Our goal is to create new methods for recognizing and studying such interactions. We hope that these methods will improve our understanding of cancer and provide a means to assess which tumors will respond to specific therapies.

Nanobiosensing Method for Point Mutation Detection of Cancer

Wang, Tza-Huei, Department of Mechanical Engineering, Johns Hopkins University 1 R21 CA120742-01A2

Description: The detection of point mutations in the DNA of tissues and body fluid has widespread implications for the study of the molecular etiology of cancer, as well as in the development of new technologies for future clinical applications. In this application, we propose to develop a clinically relevant genetic analysis technology that enables multiplex detection of point mutations in unamplified genomic DNA, using limited amounts of clinical samples. This amplification-free detection technology will be developed by using a combination of two innovative technologies, single-molecule detection (SMD) and quantum dot (QD)-mediated fluorescence resonance energy transfer (qFRET). Preliminary studies have yielded promising results, indicating that this integrative SMD-qFRET technology is able to detect DNA targets at extremely low concentrations (~5 fM), obviating the need for target amplification. When incorporated with allele-specific oligonucleotide ligation, this technology can enable the detection of low-abundance point mutations in unamplified genomic DNA. This project consists of three specific aims. First, we will develop an amplification-free point mutation detection method and evaluate it by analyzing four representative point mutations in the KRAS gene (at codon 12 and codon 13) and one commonly occurring mutation in the BRAF gene (at codon 599) in unamplified genomic DNA from ovarian serous tumors. Second, we will enhance the sensitivity and resolution of this new method to 0.5 fM and 0.5 percent (mutant:wild-type ratio of 1:200), respectively, by optimizing both the design of the QD-mediated fluorescence energy transfer system and the ligation reaction conditions. Third, we will increase the analysis throughput and mass detection efficiency of the assays by implementing this new detection method in a multiplex, microfluidic format. We will design and fabricate a microfluidic array device and use it to dispense and guide microvolumes of genomic DNA samples for multiplex analysis, using SMD spectroscopy for seven mutation assays simultaneously. It is expected that, as compared with conventional polymerase chain reaction (PCR)-based mutational analysis, this new technology will provide a more rapid and reliable measure in detecting point mutation using a 5 µg/L or less assay volume. If successfully established, it could provide a relatively straightforward molecular diagnostic platform for cancer detection and can potentially be performed in many laboratories and clinical settings.

Abscription-Based CpG Methylation Assays for Early Cancer Detection

Hanna, Michelle, Ribomed Biotechnologies, Inc. 1 R43 CA132851-01 (SBIR)

Description: This application is focused on the development of a simple and sensitive technology for the detection of CpG methylation in CpG islands of human cancer cells. CpG islands are short genomic segments in the promoter regions of most human genes that are enriched in the dinucleotide CpG. CpG islands are unmethylated in normal tissues but become progressively more methylated in cancer cells, leading to repression of genes that control the cell cycle. Most current methods for the detection of CpG methylation depend on bisulfite treatment to allow discrimination between methylated and unmethylated cytosines, followed by polymerase chain reaction (PCR) amplification of the DNA. Unmethylated cytosines are converted to uracils, while methylated cytosines remain unchanged. Chemically converted DNA is then analyzed by a wide range of methods, including sequencing or microarrays. Bisulfite treatment is technically difficult to perform, consistently causing extensive damage to sample DNA and inducing variability in these assays. The main goal of this application is to develop a methyl-CpG detection system that does not employ chemical modification of sample DNA by bisulfite treatment or PCR amplification. Signal generation will be based on Ribomed's proprietary Abscription (abortive transcription) process in which DNA detectors, called abortive promoter cassettes (APCs), are bound to methyl-CpG sites via methyl-CpG-specific probes. The proprietary Abscriptase enzyme will produce short RNA molecules from the APCs as signals for the presence of methylated CpGs. The specific aims of this project will focus on methods to produce a detection reagent composed of a methyl-CpG binding protein covalently linked to an APC in high purity and at low cost. A system for capturing unmodified single-stranded CpG islands and reconstructing their methylation patterns will be validated in conjunction with the validation of the methyl-CpG binding probes. A method for interrogating a specific individual CpG site, and a method for measuring the overall level of methylation of a CpG island, will be demonstrated. Successful completion of the proposed research will provide a simple and sensitive diagnostic method for

assessing CpG methylation in CpG islands with applications in cancer screening. The first proof of principle will focus on a segment of the p16 CpG island and CpG sites that have been intensively analyzed in the literature. Commercial products resulting from this research will be applied to cancer diagnosis, prognosis, and monitoring of treatment.

A Rapid Solution-Phase Immunoassay for Proteomics

Tyrrell, Steven Patrick, Controlled Process Technologies, LLC 1 R43 CA128780-01A1 (SBIR)

Description: Described herein is the development of the EVEIA immunoassay, which has the potential to supplant existing techniques for the validation of low-abundance biomarkers. Protein assays are a core technology of modern medicine, as nearly all diseases (including cancer) can be detected by monitoring protein concentrations in the human body (e.g., PSA, BRAC-1 and BRAC-2). However, the validation of newly identified low-abundance biomarkers (a rapidly growing list due to the Human Genome Project) is an area that is not well served by current technology. EVEIA will become the platform of choice for biomarker validation, mainly by displaying the superior sensitivity necessary for meaningful measurements of low-abundance proteins, but also by providing simpler assay development and being easier to use than other "high-sensitivity" assay platforms. Success in the validation arena should also lead to opportunities in the diagnostics market. Low-abundance proteins are hard to measure, especially in clinically relevant contexts such as plasma or saliva. This difficulty leads to a nonproductive cycle in the research and clinical arenas with regard to such proteins. Since it is difficult to determine the clinical relevance of a protein that is difficult to measure, there is little demand for instruments to measure such proteins in clinical laboratories. Conversely, with little clinical demand, there is little pressure to determine the clinical relevance of these low-abundance proteins. Unfortunately, most new protein discoveries can be expected to be found in low abundance. An instrument or assay that would make the measurement of such proteins easier would speed research and enhance clinical medicine. This application describes the basic, ongoing development of EVEIA, including comparisons with existing high-sensitivity assays. Controlled Process Technologies (CPT) has assembled a team with more than 80 person-years of laboratory and instrument development experience, authorship of nearly 100 peer-reviewed publications, and more than 15 issued patents. This team has the expertise to bring the EVEIA assay to the point that its applications in both the research and clinical laboratory are readily apparent. As specifically applied to cancer health care, the EVEIA technology has the potential to greatly expand the population of available biomarkers by allowing the validation and analysis of low-abundance proteins that can be used for the diagnosis, monitoring, and basic research of human cancer. CPT is developing a protein immunoassay and instrumentation that is expected to have a level of sensitivity two to three orders of magnitude better than existing commercial instruments while maintaining ease of use and cost benefits. Such an instrument will allow the study and detection of protein biomarkers discovered by the Human Genome Project that are normally found in concentrations too low for easy detection by current technology. Biomarkers that might be otherwise ignored could therefore become generally available for medical diagnosis and research.

Molecular Analysis of Steroid Hormone Receptors With X-Ray Crystallography

Nettles, Kendall, Florida Department of Cancer Biology, Scripps Research Institute 1 R33 CA132022-01A1

Description: The long-term goals of this project are to understand the regulation and role of steroid hormone receptors in cancer development and therapeutics through our development of a new technology that allows for the rapid analysis of steroid hormone receptors with x-ray crystallography. Specifically, our approach has demonstrated that we can increase both the rate and the numbers of hits with crystal structures by at least a thousandfold, and that this allows rapid analysis of the ligand-binding domain of these receptors bound to chemotherapy agents and pathway-selective compounds. The estrogen receptor (ER) and androgen receptor (AR) are implicated in the development, diagnosis, and treatment for breast and prostate cancer, respectively. Glucocorticoids have a broader role as upfront therapeutics for the treatment of several malignancies (e.g., leukemia and hormone-refractory prostate cancer) and as adjuvants that reduce the side effects of other chemotherapy agents. The synthetic compounds that target these receptors have, however, significant problems, including acquired resistance and undesirable side effects. They also display tissue- and pathway-

selective signaling that is poorly understood at both the molecular and structural levels. It is possible to develop tissue- and pathway-selective compounds that ameliorate some of these problems, but there is very little understanding of the structural basis for such selectivity. The lack of good structural models for tissue selectivity is due to the difficulty in producing crystal structures. The steroid receptor ligand-binding domain (LBD) has proven very difficult to crystallize because of conformational heterogeneity and protein misfolding. Here we propose to further develop our new technology for molecular analyses of steroid receptors, which we strongly believe will revolutionize the use of x-ray crystallography in both basic research and drug discovery, especially regarding steroid receptors. Specifically, we have identified and generated a series of surface mutations that stabilize the ER in the conformations seen with both agonist and antagonist ligands. This advance has allowed us to add compounds in parallel to the purified protein and to obtain the first structure of an apo steroid receptor LBD. We propose to apply this high-throughput technology to other steroid receptors implicated in cancer and to use this approach to define the structural basis through which the glucocorticoid receptor (GR) inhibits the NF-κB oncogenic pathway. We believe that these studies will establish new and robust techniques that will revolutionize the use of x-ray crystallography in defining how small molecules control tissue- and pathway-selective signaling through steroid hormone receptors. This "class analysis" approach to studying groups of structures is highly novel and allows for the incorporation of statistical power into structural analysis. Importantly, this approach will also have a direct impact on the drug discovery process by rapidly providing structural information that will guide the development of new therapeutics.

HTS for FADD Kinase Inhibitors Using Molecular Imaging

Rehemtulla, Alnawaz, Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor
1 R21 CA131859-01A1

Description: Using differential expression profiling, quantitative two-dimensional (2-D) gel electrophoresis, and data mining, we recently identified a new prognostic biomarker, Fas-associated death domain (FADD), which is overexpressed in a number of human malignancies such as lung, head and neck, and brain and adult male germ cell tumors. Studies in lung cancer revealed that overexpression of FADD is significantly associated with poor clinical outcome. Immunohistochemistry-based tissue microarray analysis confirmed the association between FADD overexpression and poor outcome and revealed the presence of nuclear localized phosphorylated FADD (p-FADD). Tumors with increased p-FADD expression also showed elevated NF-κB activation. Taken together, published results from our laboratory and others suggest a causal relationship between the phosphorylation of FADD and NF-κB activation, a hallmark of an aggressive, therapy-resistant cancer phenotype. Therefore, we hypothesize that inhibiting FADD phosphorylation in tumor cells may sensitize cancer cells to chemotherapeutic agents. To aid in experimentation of this hypothesis, we have resorted to molecular imaging tools and developed a pan-FADD kinase reporter (FKR) that noninvasively senses FADD-kinase activity in real time. In Specific Aim 1, we will characterize the sensitivity and specificity of FKR. In Specific Aim 2A, we will perform a high-throughput screen to identify molecules from a diverse set of compound libraries that target FADD phosphorylation. With secondary screens with cells expressing either mutant FKR or luciferase, the toxic and less sensitive lead molecules will be eliminated. In Specific Aim 2B, we will evaluate the relative efficacy of the candidate molecules by quantifying the 50 percent inhibitory concentration (IC_{50}) of the top leads. In Specific Aim 2C, we will investigate the specificity of candidate molecules in inhibiting FADD kinases, using Western blotting and protein kinase arrays. The utility of these compounds and their derivatives in the treatment of cancers will be investigated in subsequent years.

Improved Cancer Biomarker Detection Using Novel Air Amplifier Designs in ESI-MS

Muddiman, David, Precision Engineering Center Department and Department of Chemistry, North Carolina State University, Raleigh 1 R21 CA134250-01

Description: The identification and quantification of candidate biomarkers that are specific for a given cancer (e.g., ovarian and prostate), using tools such as liquid chromatography-mass spectrometry (LC-MS), are necessary for the diagnosis and treatment of the disease. Improved limits of detection for LC-MS and LC-tandem MS (LC-MS/MS) by implementation of devices such as the air amplifier proposed here will further these efforts, enabling better patient care. Design using computer modeling and simulation and fabrication using precision engineering methodologies will result in a stable, low-tolerance air amplifier. The improved limits of detection by a well-designed and -implemented air amplifier device will result in detection and quantification of low-abundance candidate biomarkers in biofluids for both discovery-based and targeted proteomic applications. Systematic evaluation of the new air amplifier designs will utilize electrospray ionization (ESI) MS. This will compare the original air amplifier design to the newly designed air amplifier based on the metrics of ionization time (MS analysis), limits of detection, and number of protein identifications from LC-MS/MS. After a reproducible protocol for the air amplifier is established, it will be directly applicable for biomarker discovery and target-based analysis. Multisite evaluation of the improved air amplifier is proposed to determine interlaboratory reproducibility. As a final element of this proposed research, the air amplifier design and application instructions will be disseminated on the Internet for open access.

Microfluidic Devices for Studying Cancer Signal Transduction

Levchenko, Andre, Department of Biomedical Engineering, Johns Hopkins University 1 R21 CA131920-01A1

Description: The emergence and progression of various cancers is heavily influenced by extracellular cues such as growth factors and inflammatory cytokines. The intracellular signaling pathways activated by these cues often have altered behavior and new dynamic properties, leading to neoplastic behavior. Furthermore, in a given cell, multiple signaling pathways may be activated, leading to complex interactions known as "cross-talk" and making it difficult to identify key carcinogenic pathways or to propose targeted treatment. Therefore, a comprehensive, systems-wide view of cancer signaling is required. An important barrier to achieving a truly systemic analysis of dynamic signaling behavior in cancer cells is the lack of a tool to systematically, efficiently, and reproducibly measure the immediate signaling outputs. Here we propose to develop and enhance novel microfluidic devices that can overcome this barrier. The devices contain dozens of miniature chambers that can be filled with cells. Cells in each chamber can be independently stimulated and monitored for a distinct signaling event by using conventional immunocytochemistry methods. In addition to the large number of parallel measurements that can be made in a single device, microfluidics offers precise control over experimental conditions, enhancing reproducibility and making quantification more accurate. Our existing prototypes have been tested for all major required functions and provide proof of principle of our technological approach. We propose to further develop the existing prototypes to enable very high-throughput experiments cataloging cell signaling signatures in diverse cancer cells. The main focus of this application is to increase the size and capacity of the device and supporting equipment and to demonstrate its functionality through a pilot screen of signaling in cancer cell lines and cells from malignant melanoma biopsies. These pilot screens may reveal new signal transduction-based markers for cancer diagnosis and suggest optimal chemotherapeutic targets. We anticipate that the device will become a powerful research tool to study signal transduction. Furthermore, the use of the device could potentially translate to clinical laboratories, specifically in its ability to perform multiple experiments directly on a patient's cells. We envision that clinicians will eventually be able to use the device in personalized therapy, for example, by using a small biopsy to screen for various signaling features that mark drug susceptibility or by directly testing the efficacy of chemotherapeutic agents.

Time-Resolved Fluorometric Method for Assay of Multiple Biomarkers

Szmacinski, Henryk, School of Medicine, Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore 1 R21 CA134386-01

Description: In this application, we propose the development of technology with the potential of at least 100-fold improved sensitivity over current fluorometric methods. Specifically, we integrate metal-enhanced fluorescence (MEF) with a time-resolved detection technique to achieve high sensitivity and the ability to monitor biomolecular interactions in real time. We propose to integrate MEF phenomena and a time-resolved phase modulation (PM) detection technique with surface-based assays to achieve high sensitivity and a large analyte concentration range, as well as to simplify the biochemical procedure. The new approach represents a significant advance in the fluorometric analysis of biomolecular interactions, with sensitivity comparable to that of enzyme-linked immunosorbent assay (ELISA) and a simplified sample procedure. Specifically, we will demonstrate the potential of MEF-PM technology, using a panel of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-5 (IL-5), IL-8, IL-16, vascular endothelial growth factor (VEGF), and RANTES. Currently, detection of multiple cytokines requires the use of the most sensitive detection technologies, such as ELISA, radioimmunoassay, and chemiluminescence, because of their low concentration in human blood. Within the projected work, we will perform feasibility studies on the reproducible fabrication of fluorescence-enhancing substrates, functionalization and optimization of surface chemistry, clinical assays that require high sensitivity and reduction in cost, optimization of time-resolved detection modality, and integration of these components into practical systems for clinical diagnostics and research. This will be accomplished by (1) developing a procedure for the reproducible fabrication of substrates with metallic nanostructures, (2) optimizing biomolecular immobilization on the surface of the MEF substrates, and (3) validating the MEF-PM method by using a panel of cytokines and comparing with ELISA. The proposed technology will meet requirements for high sensitivity (1-10 pg/mL), broad analytical range (4-5 orders of magnitude), ease of use, and versatility. MEF-PM will be of broad use in basic and cancer research applications and will provide a tool for proteomics, bioassay developments, and clinical diagnostics.

Novel Detector for Molecular Imaging Studies

Squillante, Michael, Radiation Monitoring Devices, Inc. 1 R43 CA134366-01 (SBIR)

Description: Microfluidic devices are a promising, emerging technology for molecular imaging studies. These chips can contain a variety of microcircuitry and microwells and are capable of manipulating nanoliter samples of reagents and solvents. The microfluidic chips have been designed for a multitude of applications, such as cell incubation studies. Adding the ability to quantify and image low amounts of radioactivity on a microfluidic chip can provide researchers with a platform to investigate molecular processes with radiolabeled probes in a controlled in vitro environment. The proposed effort will be aimed at investigating a microfludic device capable of detecting and imaging charged particles emitted by radiopharmaceuticals present in the microfluidic chamber. This high-resolution detector would be produced and investigated for in vitro molecular imaging. In addition to high spatial resolution, the proposed detector will also be capable of detecting uptake in cells at levels significantly below (by about two orders of magnitude) current, state-of-the-art in vivo imaging biological imaging systems. In view of these considerations, the proposed detector will be very promising for molecular and cellular imaging in cancer studies.

MMPA: A Novel Multiplexing Methylation Analysis Technology

Guo, Baochuan, GLC Biotechnology, Inc. 1 R44 CA134258-01 (SBIR)

Description: This fast-track SBIR project is to develop a novel multiplexed methylation profiling assay (MMPA) technology for methylation analysis of DNA derived from clinical specimens. Specifically, we will develop the MMPA assays (one for each type of cancer) for analysis of DNA methylation in colorectal, lung, and breast cancer, the three deadliest cancers. Each assay can simultaneously determine the methylation status of 20 genes in a 10,000-fold excess of unmethylated DNA. The assays can also determine the degree of methylation and the relative abundance of methylated genes in clinical specimens. In addition, the assays will be cost-effective and easy to operate. The goal of Phase I is to determine whether the MMPA assay can accurately reveal methylation in DNA derived from clinic samples. Experimentally, we will use our existing MMPA assay to profile the methylation status of eight genes in DNA derived from stools, the most complex specimen. The specific milestone is to demonstrate that this assay can achieve the detection sensitivity and specificity of 90 percent or better. Phase II is to develop the MMPA assays for analysis of DNA methylation in colorectal, lung, and breast cancers. Each MMPA assay "fires one shot" to "kill four birds." First, the assay can determine the methylation status of an individual gene; second, the assay profiles DNA methylation among a number of genes; third, the assay has the capability of determining the degree of methylation of methylated genes; and fourth, the assay provides insights into the abundance of methylated genes in clinical specimens. Clearly, these unique features will make MMPA the method of choice for methylation analysis, and thus the success of this project will have a profound impact on both cancer screening and basic cancer research.

Advanced Technology for Assaying Cancer Drug Resistance

Lim, Mark, AmberGen, Inc. 2 R44 CA114126-02 (SBIR)

Description: Significant advances have been made toward the development of a new generation of molecularly targeted cancer drugs, many of which are only now emerging from the pipeline. This project aims to develop a new, highly sensitive technology for detecting drug resistance mutations in proteins that preexist prior to treatment or are acquired due to the selective pressure exerted by treatment with molecularly targeted anticancer drugs (ACD). This problem is exemplified by drug resistance developed in patients treated for chronic myeloid leukemia (CML) with the small molecule drug imatinib (Gleevec, Glivec, STI571). It is well documented that this resistance arises from mutations in BCR-ABL tyrosine kinase, the target for imatinib. Drug resistance also occurs in patients with Philadelphia chromosome-positive (Ph+) acute lymphatic leukemia (ALL) and those with gastrointestinal stromal tumors (GIST) who are treated with imatinib. Several problems must be overcome in order to effectively detect and characterize drug resistance mutations against anticancer drugs: (1) The spectrum of mutations can be very diverse, occurring outside the drug binding pocket. In the case of the BCR-ABL kinase, mutations appear throughout the kinase domain, such as the P-loop (ATP binding) and A-loop (regulatory region). This can necessitate DNA sequencing of the entire gene or specific portions in order to detect the occurrence of both known and uncharacterized mutations. However, DNA sequencing is expensive when incorporated into a commercial assay and has limited sensitivity (>20 percent). (2) It is possible to isolate the drug target proteins or fragments to perform functional and/or structural analysis. However, such analyses are hindered by difficulties in assaying targets in crude biological mixtures and in isolating target proteins in a pure form. In this project, these limitations are overcome by using novel technology developed by AmberGen for isolating highly purified cell-free expressed polypeptide fragments of the drug-targeted protein(s) and detecting characteristic drug resistance mutations with a matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) scanning technique. The new approach, termed drug resistance assay for mutations against anticancer drugs (DRAMA-ACD), has the advantage of allowing low-cost scanning for mutations (even those previously undiscovered) with high sensitivity and high throughput. Achievement of key milestones during Phase I included (1) demonstration that DRAMA-ACD could detect mutant BCR-ABL tyrosine kinase at a sensitivity of at least 5 percent; (2) successful demonstration of PC-SNAG, a method of capturing and photoreleasing cell-free expressed fragments of the BCR-ABL tyrosine kinase with significantly reduced levels of nonspecific contamination; (3) demonstration that all steps in DRAMA-ACD can be multiplexed using bead-sorted libraries of in vitro expressed proteins (BS-LIVE-PRO); and (4) development of a process known as PC-

PRINT, which enables beads containing the target peptides to be transferred directly to a MALDI-MS target for direct analysis. During Phase II, we will continue to focus on the development and application of DRAMA-ACD to detect mutations in the BCR-ABL tyrosine kinase. An important milestone will be a demonstration of the ability to detect these mutations in CML patients with a sensitivity of 1 percent. The research will be carried out in collaboration with Dr. Adam Lerner, Associate Professor of Medicine and Pathology, a leading expert in the area of hematologic malignancies, who will provide us with samples for analysis from CML and Ph+ ALL patients undergoing imatinib treatment. All results will be statistically analyzed in collaboration with Prof. Josée Dupuis, Associate Professor of Biostatistics, and Boston University School of Public Health. We will also maintain a close contact during Phase II with two leading diagnostic companies, LabCorp and Genzyme Genetics, which have expressed an interest in the ultimate commercialization of the DRAMA-ACD approach.

Application of Emerging Technologies for Cancer Research

Objectives and Scope

This RFA is intended to support projects to evaluate the usefulness of emerging technologies in appropriate biological contexts in order to assess reproducibility and produce preliminary data toward a biological or clinical question. Technologies proposed for this RFA should be sufficiently advanced in development to be applied in a relevant clinical or biological context. (Applicants proposing to use technologies that still require significant development before they can be applied to the analysis of biological materials should consider applying to the Innovative Technologies for the Molecular Analysis of Cancer, RFA CA-05-002, http://grants.nih.gov/ grants/guide/rfa-files/RFACA05002.html.) Researchers may propose, in the R21 phase, to continue minor refinement of newly developed technologies or minor adaptations of existing technologies to new uses. The R33 phase should be used for applying the technology in a relevant setting to generate the preliminary biological data to prove that the technology functions reproducibly and effectively in the chosen biological context. These projects should provide investigators with sufficient data to demonstrate the capabilities of the technology in a biologically relevant setting. These data may then be used by the investigator or by others to propose new projects using the technology to answer biological or clinical questions, through other existing program announcements (for example, see http://www3.cancer.gov/prevention/cbrg/edrn/ and http://spores.nci.nih.gov/) or as investigator-initiated R01 applications. The data generated may also be used by technology developers in partnership with clinical or biological investigators with questions that the new technology could elucidate or to partner with industry to further refine the technology into a commercial product.

It is expected that investigators who developed successful cancer-relevant technologies under previous IMAT initiatives or under the new RFA CA-05-002 (Innovative Technologies for the Molecular Analysis of Cancer) will propose projects for this RFA. However, this RFA is not limited to technologies developed under the IMAT program. Investigators may propose to begin to utilize any emerging cancer-relevant technology. Areas of emerging technologies of interest to be applied in these projects include, but are not limited to, those technologies that enable:

- In vitro scanning for and identification of the sites of chromosomal aberrations that reflect inherited aberrations or somatic alterations resulting from aging or oxidation or exposure to radiation or carcinogens, including those that are suitable for scaling for use across whole genomes, detecting DNA adducts, or detecting rare variants in mixed populations;
- In vitro scanning for and identification of sites of mutations and polymorphisms that reflect inherited aberrations or variations, or somatic alterations resulting from aging, oxidation, or exposure to radiation or carcinogens, including those that are suitable for scaling for screening whole genomes, detecting DNA adducts, or identifying infrequently represented mutations in mixed populations of DNA molecules;
- Technologies for detection and characterization of nucleic acid sequences of novel exogenous infectious agents that may be present in human cancer;
- Highly specific and sensitive detection of specific mutations;
- Detecting mismatch and recombinational DNA repair related to cancer susceptibility and drug sensitivity;
- In vitro multiplexed analysis of the expression of genes;

- In vitro detection of expression of proteins and their modified forms, including technologies suitable for expansion to profiling of all proteins expressed in cells, detecting rare variants in mixed populations, and detecting protein adducts involved in chemical mutation;
- Monitoring the function of proteins and genetic pathways, including measurement of ligand-protein complexes and technologies for monitoring protein function of all members of a class of proteins or a complete genetic pathway;
- Delineating molecular expression, function, and analysis at the cellular level in the context of both the whole body and in situ, including molecular imaging technologies suitable at this scale, contrast agents, gene amplification techniques, and related data analysis tools;
- Technologies to elucidate molecular modifications of macromolecules that may be indicative of and critical to the transformation process;
- Delivery technologies and approaches to enable faster and more accurate delivery of molecular and cellular labels and drugs to and within cells for research and treatment with the overall goals being speed, accuracy, and biocompatibility; and/or
- Development of high-throughput, quantitative assays for epigenetic alterations (e.g., acetylation and methylation) in promoter region of genes and histone proteins isolated from biological fluids and tissues.

For all projects proposed, it will be important to substantiate the ultimate value and role of the technology in deciphering the molecular anatomy of cancer cells or analyzing the molecular profile of the individual. Inherent in this early technology application is the potential for ultimately transferring knowledge gained, technology, and/or methodology to other laboratories or the clinic. In the case of technologies intended for use on clinical specimens or in patients, applications from or collaborations with investigators involved in the clinical research of cancer are encouraged.

FY 2005

Chemotherapy With Injectable Microdroplets

D'Errico, Francesco, Department of Engineering, Yale University 1 R21 CA112144-01A1

Description: This application proposes the evaluation for chemotherapy applications of a novel drug delivery technology based on injectable emulsions of perfluorocarbon droplets containing therapeutic agents. The micron-size droplets dispersed in the emulsions are superheated (i.e., they are kept slightly above their boiling point). The droplets can be vaporized by exposure to ultrasound, which permits the spatially and temporally controlled release of their drug content into a target region. The droplets are encapsulated with surfactants protecting them from the mechanical stresses due to inoculation and circulation in the bloodstream. Therefore, they do not vaporize spontaneously, only when triggered externally. The use of ultrasound as a triggering modality enables the integration of targeted delivery and imaging. Controlled and localized release is expected to increase the therapeutic index by minimizing systemic, nonspecific exposure to the drug. The project is a collaboration between Yale University, where the superheated emulsion technology was invented and drug delivery applications were proposed, and the University of Michigan, where the use of superheated emulsions has been proposed for occlusion therapy and proven in vivo. The two groups, with complementary interests and expertise, propose to explore the capabilities of the technology in a biologically relevant setting (i.e., the transport and release in vivo of paclitaxel, a highly cytotoxic chemotherapy agent). The project will comprise investigations in various animal models to clarify different aspects of the delivery technology, as well as the utilization of various imaging techniques to confirm and quantify occurrence and location of droplet activation. Specific Aims of the R21 phase are to (1) develop the emulsion manufacturing technology, investigating and optimizing droplet size, formulation, drug loading efficiency, and ultrasound sensitivity; (2) investigate the emulsions in vitro, simulating circulation in the bloodstream, determining the ultrasound intensity required to trigger the droplets and the triggering efficiency, and verifying that anticancer drugs retain their cytotoxicity after they have been released; and (3) ascertain in vivo that droplets can circulate without undergoing spontaneous vaporization and yet be triggered to vaporize using ultrasound.

Molecular Genomics of Breast Cancer

Malkhosyan, Sergei R., Department of Cancer Genetics and Epigenetics, Burnham Institute 1 R33 CA112885-01

Description: Distortion of the cell genome characterizes neoplastic transformation. Genetic alterations that occur in tumor cells lead to activation of positive regulators of cell growth or survival and inactivation of factors that suppress these processes. A particular type of genomic alteration, chromosomal segment copy number imbalance, plays a significant role in malignant transformation: Chromosomal deletions may inactivate tumor suppressor genes, while chromosomal segment amplifications may increase the gene dosage of oncogenes. In this study, we propose to apply a new technique, Comparative Hybridization of AP-PCR Arrays (CHAPA), which was developed in our laboratory, for high-resolution profiling of breast tumors for DNA copy number alterations. This will allow the detection of single DNA copy number losses or gains at thousands of sites throughout the genome of the cancer cells (Specific Aim 1). We hypothesize that such genetic signatures may embrace the information on what cancer genes were responsible for the development and progression of each tumor and, consequently, the resulting pathologic behavior of tumor cells and their responsiveness to treatment. This general hypothesis will be tested by the analysis of genetic profiles to differentiate breast tumors according to their pathways of tumorigenesis (known or novel) and by the analysis of genetic profiles of breast tumors in association with their clinicopathologic characteristics, recurrence, and patient's survival to reveal genetic markers for cancer diagnosis and prognosis. Once frequent (common for independent tumors) genomic alterations have been identified, they will be compared with the loci known to play a role in breast cancer development. The genetic aberrations in chromosomal regions that do not contain known cancer genes will be selected for further characterization, with the ultimate goal to identify the underlying novel cancer genes (Specific Aim 3). These experiments will provide a comprehensive view of the role of genetic aberrations in breast tumorigenesis. They will also help to identify genetic markers for breast cancer diagnosis, development, and prognosis and facilitate the identification, mapping, and eventual isolation of novel cancer genes.

Enhancement of Brain Tumor Immunotherapy by Fas-L RNAi

Olivi, Alessandro, Department of Neurosurgery, Johns Hopkins University 1 R21 CA112148-01

Description: Each year, approximately 185,000 people in the United States are diagnosed with a primary or metastatic brain tumor constituting the third leading cause of death in young adults ages 20-39. Among brain tumors, malignant gliomas are the most common and aggressive malignancies. Gliomas seem to be capable of inducing T cell apoptosis through the Fas/Fas-ligand (Fas-L) pathway. This mechanism allows them to circumvent immune surveillance by decreasing cell-mediated immunity. Brain tumor therapy using immune modulators such as interleukins (ILs) increases the recruitment of active T lymphocyte populations and has proven to be an effective strategy in experimental models of the disease. However, due to tumor-secreted Fas-L, the peritumoral T cells recruited by IL are activated through the transmembrane Fas receptor, which initiates the caspase-3-mediated apoptotic cascade. Using RNA interference (RNAi) techniques, mRNA from tumor-derived Fas-L could be silenced, and T cell apoptosis could be decreased, thus improving antitumor responses and potentiating the effect of IL therapy. RNAi sequences can be delivered by retroviruses, guaranteeing a constitutive transfection. In this proposal, the effect that treatment with Fas-L RNAi sequences delivered via retroviruses has on experimental gliomas will be investigated. The effect of this treatment modality will be studied alone and in combination with locally delivered IL incorporated into injectable microspheres. Treatment with Fas-L RNAi sequences is expected to decrease levels of tumor-derived Fas-L, therefore decreasing the rates of T cell apoptosis and increasing the populations of peritumoral T cells. Such an effect should allow a more consistent cell-mediated antitumor response able to prolong survival in animal models. Furthermore, the efficacy of locally delivered IL microspheres should be enhanced. The potential benefit of Fas-L RNAi delivered in this fashion could be applicable to several malignancies that have the Fas/Fas-L pathway among their immune privilege strategies.

How Do Colorectal Cancers Arise Despite Surveillance?

Shibata, Darryl K., Pathology Department, University of Southern California 1 R33 CA111940-01

Description: Colorectal cancers are widely believed to develop through an adenoma-cancer sequence. By this paradigm, all cancers should be preventable with surveillance and polypectomy. However, in most surveillance studies, some cancers inevitably appear only a few years after negative clinical examinations. It is uncertain how such "interval" cancers appear, but either an adenoma was missed during the last "negative" examination, or there was an unexpected "rapid" mode of progression. A large number of such interval cancers have been found during an ongoing clinical surveillance program of high-risk individuals with germline mutations in DNA mismatch repair (MMR) genes or hereditary nonpolyposis colorectal cancer (HNPCC). These interval cancers provide unique opportunities to rigorously understand why prevention fails. The key emerging technology is a new capability that infers time from cancer mutations. A molecular tumor clock can quantitatively infer times since MMR loss, and ages of final cancer expansions—the more mutations in a cancer, the greater these intervals. Distinguishing between failure from inadequate surveillance ("missed adenomas") and failure due to the unique biology of HNPCC colorectal cancers ("rapid histologic or genetic progression") is critical for the development of more effective strategies to prevent and treat colorectal cancer.

Peptide Profiling Techniques to Detect Thyroid Carcinoma

Tempst, Paul J., Sloan-Kettering Institute for Cancer Research 1 R21 CA111942-01

Description: The information required for adequate diagnosis, treatment, and monitoring of cancers is so complex that a panel of measurements, used in sum, may provide the best answers. The concept is embodied in SELDI-TOF mass spectrometric (MS) peptide profiling, an emerging technique for serum-based cancer detection. Even though SELDI has thus far produced only low-complexity spectra, the patterns, when analyzed as groups, have the potential to create learning algorithms with diagnostic accuracies as good as or better than conventional biomarkers. We have developed a system to capture peptides on magnetic reversed-phase beads, followed by MALDI-TOF MS, to yield increasingly complex, yet very reproducible patterns. This has clear advantages, as more displayed peptides provide more opportunity to select unique patterns (barcodes)

for cancer subtypes and stages and to predict and monitor clinical outcome. Extreme care has also been taken to standardize specimen collection, handling, and storage to avoid the introduction of artifact. Pilot projects at MSKCC with a variety of malignancies suggest that peptide patterns thus obtained appear to hold information that may have direct clinical utility. The goals of this project are to (1) automate our prototype serum peptide profiling platform and implement machine learning methods that use the resulting peptide patterns (barcodes) for sample classification [R21] and (2) test the barcode diagnostic model in a high-throughput setting, using well-defined and carefully observed groups of thyroid carcinoma patients [R33]. R21 Aim 1 is to automate serum sample processing and analysis; Aim 2 is to automate all data processing, examine pattern selection and sample class prediction methods, and integrate all software platforms; and Aim 3 is to develop routine MALDI-TOF/TOF tandem MS sequencing of barcode peptides. R33 Aim 1 is to define reproducibility of serum patterns in patients with thyroid disease; Aim 2 is to determine barcodes that can distinguish patients with thyroid cancer from those with benign thyroid nodules; and Aim 3 is to assess whether serum peptidome barcodes can identify occult metastasis in a large group of thyroid cancer survivors.

A High-Throughput Diagnostic Assay for Lung Cancer

Brown, Kathlynn C., Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas 1 R21 CA114157-01

Description: Within the United States, 170,000 new cases of lung cancer are diagnosed per year. Over 60 percent of these patients will die within 1 year, making lung cancer the largest cancer killer of both men and women. The correct histopathological diagnosis of a tumor is critical in determining the appropriate treatment. However, precise classification of tumors remains a significant biomedical challenge. Furthermore, tumors of similar histology can have different clinical outcomes, stressing the need for more detailed molecular classifications. Generation of ligands specific to receptor(s) on the surface of a lung cancer cell will impact clinical issues, including functional diagnosis. Our overall goal is to generate a panel of cell-specific molecules that could be used to classify tumor types and utilize these cancer-specific reagents in a high-throughput diagnostic assay. Using phage display technologies, my laboratory has developed platform methodologies to isolate peptides that bind to and mediate uptake into specific cell lines. We have identified cell-specific targeting peptides for 25 different cell types, including 4 lung cancer cell lines. The isolated peptides display remarkable cell specificities, even among similar cells, and are able to discriminate between normal and cancerous cells as well as different lung tumor cells. This high discriminating power suggests that peptides could be identified that selectively bind to different tumor types, even those with similar classifications. We propose to expand this panel of lung-specific reagents by isolating cell targeting peptides for four different lung cancer lines and then utilize these peptides as diagnostic reagents. These peptides will be assayed for affinity and cell specificity. We will remove the peptides from the phage backbone and synthesize peptide scaffolds that retain their affinity and cell specificity. We will develop a high-throughput fluorescent assay based on peptide binding that will allow for a more molecular classification of lung cancer samples. The assay can be multiplexed so that multiple binding events can be examined on a single sample. At the end of this pilot project, we will have developed a novel diagnostic platform that can be expanded to clinical samples. Furthermore, the technologies developed here can be applied to other forms of cancer.

Typing the Transcriptome in Cancer Using Splicing Array

Fu, Xiang-Dong, Department of Cellular and Molecular Medicine, University of California, San Diego 1 R33 CA114184-01

Description: Alternative splicing is a permanent feature in higher eukaryotic cells, and understanding how alternative splicing alters the composition and function of the proteome represents a major challenge in the postgenome era. For cancer research, unique mRNA isoforms may provide a robust set of biomarkers for diagnosis and prognosis, and cancer-specific mRNA isoforms may serve as discriminating targets for effective therapeutic interventions. Furthermore, understanding how splice choice is made and regulated in development and disease is a fundamental issue in cancer cell biology. An mRNA isoform-sensitive microarray technology would be ideally suited and timely for addressing a wide range of clinical and mechanistic questions regarding

alternative splicing. In the past IMAT funding period, we have developed a unique and novel technology platform to attack the splicing problem. After a systematic and substantial effort in database construction and experimental development, the technology has matured, and its superiority in reproducible measurement of mRNA isoforms under a variety of conditions has been demonstrated. Unique to the splicing array is the need to progressively enlarge the database for accurately annotated mRNA isoforms and prepare corresponding oligo sets for measurement. We are therefore seeking IMAT support to put the technology into practical use and let the research community take advantage of the technology development.

We have three specific goals for the next phase in applying the emerging technology for molecular analysis of cancer: (1) We plan to use the technology to identify unique mRNA isoforms associated with prostate cancer. We will survey existing prostate cancer cell lines untreated or treated with androgen and estrogen as well as cancer tissues at different malignant stages to identify tumor-specific and hormonal regulated alternative splicing. (2) We propose to apply the technology to address mechanisms of splicing regulation by identifying direct targets for a large number of splicing regulators in knockdown and knockout cells. (3) Along with the proposed technology applications, we will progressively enlarge the high-quantity databases coupled with the technology development and continue to improve and enlarge the database by adding new features and functions and develop linked software for splicing array data analysis.

Imaging Tumor-Associated Fibroblast Activation Protein

Tung, Ching-Hsuan, Massachusetts General Hospital 1 R21 CA114149-01

Description: The long-term goal of this research is to develop a novel fibroblast activation protein (FAP) sensing near-infrared fluorescence reporter for early tumor detection and tumor classification. FAP is a cell surface antigen of reactive tumor stromal fibroblasts found in more than 90 percent of epithelial carcinomas, but it is absent from epithelial carcinoma cells, normal fibroblasts, and other normal human tissue. Supporting tumor stromal fibroblasts are generally localized close to tumor vasculature, which is essential for early tumor development and growth. Thus, it has been chosen as a target for monoclonal antibody-based tumor therapy. FAP is not only a membrane protein but also a dipeptidyl peptidase. An imaging probe to report enzymatic activity and location of FAP could be extremely useful for early tumor detection. In this application, we will develop a small molecular probe with ultrasensitivity based on a unique class of fluorogenic chromophore that has significant changes in emission at different chemical states. Specifically, the probes emit no fluorescence in their initial intact state but become brightly fluorescent after specific proteolytic reaction. The newly developed low-molecular-weight, well-defined fluorogenic probes are expected to have several advantages for imaging: (1) fast tissue distribution allowing earlier imaging after injection, (2) fast clearance allowing repeated imaging, and (3) high likelihood of developing key candidates into clinically useful agents. The choice of FAP is based on its importance in tumor growth, invasion, and other processes in oncogenesis. Together with recent developments in fluorescence imaging technologies, this research is expected to ultimately result in clinical imaging agents with specificity for targeted enzymes. We believe that the developed approach can be used as a platform to design a broad spectrum of activatable molecular probes to image other amino peptidases in vivo.

Identification of Autoantigens in Ovarian Cancer

Philip, Ramila, Immunotope, Inc. 1 R43 CA114194-01 (SBIR)

Description: Early diagnosis and immunotherapeutic stimulation of a patient's own immune system to detect and destroy tumors constitute the best hope for the treatment, prevention, and eventual cure of ovarian cancer. The overall goal of this proposal is to conduct proof-of-concept studies to identify tumor-associated antigens (TAAs) that will lead to the development of (1) early-stage diagnostics and (2) immunotherapeutics that induce strong cellular (T cell) and humoral (B cell) responses against ovarian tumors. The novelty of this program is in the identification of tumor-specific antigens that are both reactive to autoantibodies in the serum of ovarian cancer patients at progressive stages of disease and processed through the immune system major histocompatibility complex (MHC) class I pathway and recognized by cytotoxic T lymphocytes. Discovery and preliminary evaluation of the antigens will begin in Project Phase I. Aim 1 of this Phase I proposal is to conduct a comprehensive proteomic analysis to identify tumor-associated antigens reactive to serum immunoglobulin from primary ovarian cancer patients at different stages of disease, with the goal of identifying antigens common to the majority of patients sampled. Aim 1 will be achieved by immunoprecipitation of TAAs from primary tumor lysates using patient serum as the source of autoantibodies and control serum to identify antigens found only in patient serum. TAAs will be fully characterized by mass spectroscopy, and a TAA proteomics database will be generated. Aim 2 is to identify the epitopes in these TAAs that are recognized by the autoantibodies in cancer patient serum. Since native conformation and posttranslational processing can have profound effects on antibody recognition, we will further characterize glycosylation patterns on these antigens and determine whether they play a role in antibody recognition. This emphasis on native proteins has important implications for our future work on the design of diagnostics and immunotherapeutics, as it takes into account the specificity of the interactions between native antigens and autoantibodies.

Technology to Optimize scFvs for Targeting Therapeutics

Federspiel, Mark J., Mayo Clinic College of Medicine, Rochester 1 R33 CA112070-01A1

Description: Antibodies provide superior targeting capabilities to a variety of therapeutic agents. Several technologies have greatly facilitated the initial identification of a variety of antibody reagents, including scFv and Fab antibodies, with virtually any possible specificity. However, lead antibodies often require further optimization to maximize their therapeutic performance: optimization of antibody expression and folding in relevant cells and optimization of the affinity of the antibody for the target antigen. The development of promising targeting antibodies against cancer often languishes at this bottleneck. Therefore, technologies to facilitate antibody adaptation and optimization are urgently needed. Antibody optimization is best achieved by the randomization and subsequent selection of antibody mutants for the desired phenotypes, since efficient rational design of antibodies is currently not feasible. Polypeptide display (e.g., phage display) is a powerful technology for the generation and screening of libraries of mutant polypeptides, for a phenotype. A eukaryotic display technology that employs the efficient protein synthesis and quality control system of eukaryotic cells would best optimize the therapeutic parameters of targeting antibodies. We have recently demonstrated the feasibility of a retrovirus, avian leukosis virus (ALV), as a viral platform for the display of a variety of eukaryotic polypeptides, including scFvs, and the efficient generation and selection of a peptide library in eukaryotic cells. The goal of this R33 application is to demonstrate the efficiency of using the ALV display technology for the optimization of the scFv scaffold for efficient folding and expression in eukaryotic cells and for generating a panel of scFvs with a range of affinities for their target antigen with an optimized scaffold. We will use the ALV display technology to optimize two scFvs with known specificity for tumor neovasculature: an antilaminin scFv (L36) that inhibits angiogenesis in a variety of assays, presumably due to the exposure of laminin in the extracellular matrix during tumor neovessel formation, and an scFv that recognizes a VEGF:receptor complex (LL4) specific to endothelium in tumor neovessels. The ability of the nonoptimized and the optimized scFvs to target a therapeutic agent to tumor neovessels will be assessed using oncolytic measles viruses. Specifically, we aim to (1) create ALV display libraries of L36 and LL4 scFv mutants by error-prone PCR, (2) screen the ALV display libraries of scFv mutants to generate a panel of L36 and LL4 scFv mutants with a range of known affinities (from fM to nM) for their target antigen and with an optimized scFv scaffold, and (3) generate

recombinant measles viruses displaying nonoptimized and optimized targeting scFvs and compare them with respect to ease of production, efficiency of scFv display, particle-to-infectivity ratios, replication kinetics, and homing properties to tumor neovessels.

Mapping Regulatory Pathways in Cancers

Green, Roland D., Nimblegen Systems, Inc. 1 R21 CA116365-01

Description: We propose to apply the emerging technology of chromatin immunoprecipitation on microarrays (ChIP chips) to identify the direct targets of transcription factors (TFs) known to be involved in breast and colon cancers. During the R21 phase of the project, we plan to identify these targets using a newly developed set of oligonucleotide microarrays that contain 15 million 50mer probes that tile through the nonrepetitive sequence of the human genome at 100 bp resolution. We will validate that our ChIP chip protocols work with this new tiling array set. We will also develop and refine two new protocols, microarray reuse and four-color hybridization, that will allow economical use of this tiling array set so that it will be a more practical tool for research laboratories. In the R33 phase of this project, we will use this tiling array set to identify the direct targets of nine TFs known to be involved in breast and colon cancers. Once we have collected the direct targets of these TFs, we will develop custom arrays that have probes that tile through all of the binding sites in the direct targets. We will use these TF-focused screening arrays to study binding patterns in Icelandic breast and colon cancer samples to determine whether TF binding patterns could be a useful means of classifying tumors.

Reversible Promoter-Insertion for In Vivo Studies of Melanoma

Kandel, Eugene, Department of Molecular Genetics, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University 1 R21 CA116060-01

Description: It is commonly accepted that the processes underlying the development of cancer are best modeled in vivo. A plethora of methods have been developed for genetic dissection of biological processes in somatic cells in culture; however, the adaptation of these techniques to in vivo use has been very limited. Our earlier experience indicates that reversible promoter-insertion mutagenesis offers a number of advantages over the currently used forward genetics techniques for cultured cells. The most important benefits are the dominant nature of the mutations and the ease of identifying the affected gene and establishing the causal link between the mutation and the phenotype. The principal limitation for the adaptation of this technique to in vivo studies is the delivery of the insertional mutagen (typically, a retroviral vector) to the desired cell type. On the basis of our recent experience and published data from others, we propose a method to circumvent this limitation via the use of specially engineered transposon vectors. We propose to apply our technique to study the genetic events underlying melanoma development. We will screen for genetic events that cooperate with oncogenic Ras (commonly found in melanomas) in this process. We will confirm the properties of the proposed insertional mutagens in culture and then proceed to establish mouse strains suitable for mutagenesis. Upon verifying the properties of our constructs in vivo, we will selectively mutagenize melanocytes in transgenic animals and will test the functional link between tumorigenesis and the inserted promoter. Confirmed hits will be the subject of future investigation, while the technique itself will become available for dissection of various biological phenomena in general and aspects of tumorigenesis in particular.

Global DNA Methylation Profiles of Head and Neck Cancers

Lizardi, Paul M., Pathology Department, Yale University School of Medicine 1 R21 CA116079-01

Description: Head and neck cancer is a common disease worldwide, and more than 40,000 cases are diagnosed annually in the United States. The 5-year survival rate is roughly 50 percent and has not improved over two decades. This research project focuses on the development of a new cancer biomarker paradigm based on the global epigenetic status of head and neck tissues. In many laboratories, DNA methylation is being examined as a means for early diagnosis of cancer. Recently DNA hypomethylation has been found to be a promising

biomarker for the more advanced and aggressive stages of cancer, and there is an urgent need for analytical tools that will sample comprehensively the abnormal "methylome" hidden within the vast landscape of DNA repeats, in addition to the abnormal methylome of promoter-associated CpG islands. This grant application focuses on the validation of a novel microarray-based approach for epigenetic biomarker discovery and cancer classification that is complementary to other methods currently in use. The new method is relatively simple and has the unique capability to sample the methylation status of the majority of DNA repeats, as well as gene promoters, generating very large datasets. We will perform microarray-based methylation profiling using a statistically meaningful set of tumor samples from head and neck cancer patients. In addition, we will utilize the microarray-generated methylation profile information to identify clinically relevant subtypes of head and neck cancer, using a variety of analytical approaches. We will refine a subtype classification algorithm by inclusion of additional datasets available for the same samples, including allele gains and losses revealed by array-CGH and HPV infection status, as well as relevant patient clinical data. We will use this new information to address urgent diagnostic and prognostic clinical needs for improved classification of head and neck cancers in relation to diagnosis of early developmental stages, assessment of aggressiveness, likelihood of metastasis, and risk of recurrence after surgery.

Application of LC-MS/MS to Formalin-Fixed HNSCC Tissue

Krizman, David B., Expression Pathology, Inc. 1 R43 CA116217-01 (SBIR)

Description: The objective of this Phase I proposal is to evaluate the usefulness of the emerging technology of mass spectrometry for proteomic analysis of formalin-fixed cancer tissue for initial application to the discovery/analysis of cancer biomarkers. The model system chosen for this proposal is head and neck squamous cell carcinoma (HNSCC). Because HNSCC is directly related to tobacco use, this proposal will be an excellent model to demonstrate cancer biomarkers that can be linked to tobacco-induced cancer. This Phase I evaluation will be accomplished by integrating the technologies of tissue microdissection, *Liquid Tissue®* methodology, and higher order mass spectrometry to demonstrate application for high-content proteomic analysis of the cancer microenvironment of formalin-fixed archival cancer tissue. Laser capture microdissection (LCM) will be employed to separately obtain normal and tumorigenic epithelium directly from formalin-fixed archival HNSCC tissue blocks. In addition, normal- and tumor-associated stromal components supporting these epithelial regions will also be obtained by LCM and analyzed in parallel. A searchable database will be developed based on mass spectrometric analysis of normal, tumor, and associated stroma from five separate HNSCC cases and bioinformatics conclusions drawn to demonstrate the ability to discover biomarkers of cancer directly from formalin-fixed archival HNSCC cancer tissue. In addition, it is hypothesized that analysis of stromal components will lead to discovery of stroma-associated biomarkers that could potentially develop a role for surrounding stroma in the diagnosis and treatment of cancer. If this demonstration Phase I is successful, a Phase II proposal will be submitted to apply this platform to discovery of proteins critical to HNSCC, resulting in commercial applications to the discovery/analysis of biomarkers of not only HNSCC but all cancers. Additional commercial applications that might result from a Phase II are (1) formalin-fixed tissue-based mass spectrometry contract service, (2) development of an internal cancer biomarkers discovery core for license to the biopharmaceutical industry, and (3) a proteomic contract service for analysis of animal models of toxicology with strategic biopharmaceutical partners. The following Specific Aims will be addressed in this Phase I proposal: (1) Assess the ability of mass spectrometry to reproducibly profile the proteome by analysis of *Liquid Tissue®* protein preparations obtained from different and distinct histological regions of formalin-fixed, paraffin-embedded HNSCC tissue; (2) assess the ability to use mass spectrometry data to develop a database reflecting proteomic

similarities and differences between normal and tumorigenic HNSCC epithelium; and (3) assess the ability to use mass spectrometry data to develop a database reflecting proteomic similarities and differences between stroma surrounding normal epithelium and stroma surrounding tumorigenic epithelium of HNSCC tissue.

Affinity Maturation of Anti-PSCA Antibodies

Takeuchi, Toshihiko, Bioren, Inc. 1 R43 CA116053-01 (SBIR)

Description: Prostate cancer is one of the most commonly diagnosed cancers in men, accounting for over 30,000 deaths annually in the United States. The current lack of predictive tests and treatment methodologies illustrates a need for improved detection, prognostics, and therapies. The goal of this proposal is to generate improved antibodies against prostate stem cell antigen (PSCA) to facilitate enhanced diagnostics and immunotherapeutics. PSCA is a prostate-specific membrane protein with increased expression correlating with prostate cancer progression and metastasis. Anti-PSCA antibodies are of diagnostic utility and, in preclinical animal models, able to prevent prostate cancer metastases and inhibit tumor growth. These findings make PSCA a great candidate for antibody-based therapeutics. For potential human applications, anti-PSCA monoclonal antibodies have been humanized through complementarity determining region (CDR) grafting, which, however, exhibits more than a 50 percent loss in affinity for PSCA. Bioren's patented proprietary Look-Through Mutagenesis™ (LTM) and Walk-Through Mutagenesis™ (WTM) technology is based on synthetic oligonucleotide chemistry to create defined antibody mutations. Using Bioren's LTM™/WTM™ strategies, an efficient, high-throughput mutagenesis system can systematically explore the chemical modalities involved in the PSCA antigen binding pocket. The advantages of our approach allow a hypothesis-driven rational replacement of codons necessary to determine and optimize amino acid functionality in all the VH and VL CDRs of the antibody. By focusing on generating targeted small diversity libraries for screening, our process is both accelerated and economically efficient, unlike other mutagenesis strategies. In combining mapped LTM/WTM beneficial mutations through an iterative permutation process, we have been able to achieve 1,000-fold (low pM KD) affinity improvements. The technology developed under this proposal can be applied in the affinity maturation of many other immunotherapy antibody candidates.

FY 2006

Targeted Antigen Delivery for Cancer Immunotherapy

Frankenburg, Shoshana, DAN Immunotherapy, Ltd. 1 R21 CA114160-01A1

Description: Metastatic melanoma patients currently have a dismal diagnosis, and treatment at the metastatic stage is generally ineffective. The long-term objective of this research project is to develop a novel treatment and vaccination approach for cancer in general and melanoma in particular, based on immunotherapy. Ex vivo antigen delivery for immunotherapy is laborious and expensive and is thus not affordable to many of those in need. The investigators propose to develop an antigenic entity that can be applied on the skin, with direct antigen delivery to skin dendritic cells and without the need for in vitro cell manipulations. Thus, the major practical objective of this study is to establish the proof of principle that topically delivered tumor-associated antigens can elicit effective antitumor responses and can be used for cancer immunotherapy.

Specific Aims: The study will be based on two antigenic proteins derived from melanoma: The first is a hydrophilic recombinant gp100 protein, and the second is a multiepitope polypeptide that comprises 3', repeats of four HLA-A2 melanoma peptides derived from three different melanoma proteins. In order to allow and to improve topical transdermal delivery, the antigens will be genetically fused to potential carrier molecules. One of these is *E. coli* heat-labile enterotoxin, a molecule recently shown to act as carrier and adjuvant. Another is a novel haptotactic C-terminal fibrinopeptide (Haptide). During the first phase of the project, R21, the new antigenic entities will be cloned, expressed, and purified. Novel in vitro models using human skin will be used to evaluate transcutaneous passage of molecules, Langerhans cells activation and mobilization, and stimulation of specific cytotoxic T cells. The rationale for the milestones that will determine continuation to the second phase, R33, is based on the efficacy of antigen delivered transcutaneously to stimulate the immune system in human in vitro models and will allow for the selection of the molecules that will be further evaluated in depth in in vivo models. In the R33 phase, specific immune responses of splenic T cells from vaccinated mice will be evaluated, tumor models will be established in mice, and the response to vaccination will be determined. Finally, the most effective molecule(s) will be produced under GMP or GMP-like conditions for Phase I/II clinical trials in a subsequent study.

Public Health: The success of this project would allow topical application of an immunostimulant for treatment of melanoma and other cancers and would thus significantly simplify treatment, eliminating the need for hospitalization and even day care and without the need for a specialized laboratory. As a result, one could treat a much larger number of patients, with the potential to clinically evaluate new antigens and immunotherapeutic modalities, improving the life quality and expectancy of metastatic melanoma patients.

Technology/Map Endothelial Targets/Human Renal Tumors

Schnitzer, Jan, Sidney Kimmel Cancer Center 1 R33 CA118602-01

Description: The molecular complexity and in vivo inaccessibility of most tumor cells within solid tumors can greatly limit genomic- and proteomic-based discovery of useful targets for tumor-specific imaging and therapeutic agents in vivo. To overcome endothelial cell (EC) barriers and achieve more effective targeting and penetration into solid tumors, we shift analytical focus from the tumor cell to the vascular EC surface and its caveolae in direct contact with the circulating blood. To reduce data complexity to a meaningful subset of targetable proteins expressed on the EC surface, we will use tissue subcellular fractionation, novel multimodal mass spectrometric analysis, in silico subtraction, and bioinformatics interrogation of structure and function to unmask, from the >100,000 proteins in the tissue, those few intravenously accessible proteins differentially expressed on vascular endothelium in human renal tumors. This technology and overall approach have been validated in rodent solid tumors whereby new vascular targets have been uncovered permitting tumor-specific imaging, penetration, and effective radio immunotherapy (*Nature*, 429:629-35, 2004). But, currently very little is known about the expression of proteins in tumor neovascular endothelium, especially in human tissue. We

now wish to apply our new technology to map comprehensively the proteome of luminal EC surfaces and caveolae in human renal tumors in vivo. It is likely that human tumors will express a different constellation of proteins on tumor neovasculature not yet uncovered or induced in animal models. To this end, we propose the following Specific Aims: (1) use novel tissue subfractionation and proteomic analytical approaches to map comprehensively vascular EC surfaces and caveolae in human renal tumors vs. matched normal renal tissue to unmask candidate tumor-induced/associated vascular proteins and (2) create new antibodies to newly discovered human renal tumor EC targets and use antibodies as probes to validate the expression of tumor-induced/associated proteins at the EC surface and its caveolae in human tissues and thereby assess the degree of target specificity for the neovasculature of human solid tumors. Such mapping may also elucidate the effects of the tumor on the developing vascular endothelium and yield important tumor-specific vascular targets for improving noninvasive diagnostic imaging and therapy as well as yield new diagnostic and prognostic markers for the molecular classification of tumor biopsies.

miRNA Profiling in Fixed Cancer Samples

Brown, David, Ambion, Inc. 1 R44 CA118785-01 (SBIR)

Description: During Phase I of our proposed research, we will develop and validate procedures for recovering, labeling, and analyzing miRNAs from fixed tissue samples. The procedures will be based on the miRNA microarray and fixed tissue RNA isolation systems that we developed in other SBIR-funded programs. The development of our miRNA isolation and labeling procedures will be accomplished using a model system wherein mouse organs will be split, with half flash-frozen and the other half formalin-fixed using a procedure that is commonly employed in hospitals. The frozen and fixed samples will be processed to recover the miRNAs. The miRNAs from the fixed and frozen tissues will be independently labeled and analyzed using miRNA microarrays. The isolation, labeling, and hybridization procedures will be varied until the fixed samples yield the same miRNA expression profiles as the equivalent frozen samples. The fixed sample procedures will then be used to analyze formalin-fixed, human tissue samples to analyze miRNA profiles from multiple organs. The fixed-tissue miRNA profiles will be compared to the profiles generated from frozen samples to verify that the fixed-tissue miRNA profiling process can be used for stored, human fixed-tissue samples. During Phase II of our research project, we will use the miRNA isolation, labeling, and microarray analysis procedures developed during Phase I to analyze archived, fixed human cancer tissues to identify miRNAs with expression profiles that are significantly different from equivalent, normal tissues. The most interesting miRNAs or miRNA signatures might provide opportunities for diagnostic/prognostic assay development or even an intervention point for therapeutic agents.

Improving Efficacy of Antitumor mAbs Through Glycoengi

Sethuraman, Natarajan, GlycoFi, Inc. 1 R43 CA118539-01 (SBIR)

Description: With antibody-based drugs emerging as a powerful new source for cancer treatment, the ability to create specifically targeted, highly efficacious antibodies quickly and cost-effectively is vital to improving patient outcomes. GlycoFi's novel yeast-based protein production platform is an emerging technology primed for development and delivery of clinical glycoprotein-based therapeutics. This project seeks to apply GlycoFi's technology toward the production of a known cancer therapeutic with the long-term objective of bringing new antibody-based cancer drugs to the clinic. The influence of the structure of Asn297 linked oligosaccharides on IgGs is well known with elements such as pharmacokinetic stability, antibody mediated cell cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC), all affected by glycosylation. Through highly targeted metabolic engineering of the *Pichia pastoris* glycosylation pathway, GlycoFi has developed unique strains of yeast capable of secreting monoclonal antibodies with homogeneous, human glycostructures. Phase I explores the feasibility of producing three different human glycoforms of anti-CD20 mAb in GlycoFi's "humanized" yeast. In Aim 1, the gene encoding anti-CD20 mAb will be cloned and expressed in strains of *P. pastoris* to produce three distinct glycoforms. The functionality of these glycoforms will be tested in vitro and compared to the commercially produced anti-CD20 mAb rituximab. Aim 2 looks at antibody binding to CD20-presenting Raji cells. Both ADCC and CDC have been implicated as mechanisms by which rituxan exerts its antitumor effect. As activation of ADCC requires antibody binding to Fcy receptors, Aim 3 analyzes the potential in vivo

efficacy of these antibodies through in vitro receptor binding assays. Activation of the complement pathway requires antibody binding to C1 q; thus, Aim 4 focuses on comparing C1 q binding abilities of the different GlycoFi-produced antibodies with rituximab. Phase II will focus on proving the clinical potential of GlycoFi's anti-CD20 mAbs through ADCC and CDC assays and in vivo pharmacokinetic and efficacy studies. With respect to relevance to public health, GlycoFi's technology allows unprecedented control over glycosylation, providing the ability to improve the biological function of therapeutic proteins including monoclonal antibodies used for cancer treatment. In addition, *P. pastoris* is a robust protein expression host, secreting large volumes of therapeutic drugs that will translate into increased speed to the clinic and lower patient costs.

Quantitative Proteomic Analysis of Lymphoma Transformation

Elenitoba-Johnson, Kojo, Department of Pathology, University of Utah School of Medicine
1 R33 CA112061-01A2

Description: Non-Hodgkin's lymphoma accounts for approximately 50,000 new cases of cancer annually. This figure represents an increase beyond that seen for most other forms of cancer. Among the non-Hodgkin's lymphomas, follicular lymphoma represents the most common subtype of low-grade B cell lymphoma in adults and typically pursues an indolent clinical course. In a significant proportion of cases there is histologic transformation from a low-grade neoplasm to an aggressive diffuse large B cell lymphoma with significantly decreased median survival. The recent advent of sophisticated mass spectrometry technology, coupled with software algorithms that permit instantaneous protein identification, makes it feasible to study the pattern of protein deregulation that distinguishes two biologic states. We propose to employ a combination of chromatographic techniques and tandem mass spectrometry in the identification of the alterations in protein expression that accompany histologic transformation. We shall be analyzing a cohort of matched pairs of follicular lymphoma and their transformed diffuse large B cell lymphoma counterparts occurring in the same individual. With respect to relevance, comprehensive identification of the qualitative and quantitative changes in protein expression that are involved in follicular lymphoma transformation will permit the delineation of deregulated pathways, identify distinct prognostic subgroups of transformed lymphoma, and facilitate the development of novel therapies that target susceptible elements in the deregulated pathways.

Applications of "Recombomice" for Cancer Research

Engelward, Bevin, Biological Engineering Division, Massachusetts Institute of Technology 1 R33 CA112151-01A2

Description: Each time a cell divides, billions of base pairs of information must be accurately copied in the face of an onslaught of DNA damage. Homology-directed repair (HDR) provides one of the most important mechanisms for coping with damaged DNA. If coding information is missing or corrupted, HDR can extract sequence information available elsewhere in the genome. Although HDR is generally beneficial, transfer of genetic information is risky, since misalignments can lead to tumorigenic rearrangements. To investigate the process of HDR in vivo, we have engineered the first mouse model in which HDR can be detected in somatic cells by the appearance of a fluorescent signal. In the fluorescent yellow direct repeat (FYDR) recombomice, recombination at an engineered substrate yields fluorescence. Recombination assays are simple and rapid, making it possible to do in days what used to take weeks. In addition, the FYDR mice overcome limitations of previous systems. For example, although APRT+/- mice can be used to detect loss of heterozygosity, technically demanding assays are necessary to identify HDR events; in the pun mice, only embryonic recombination events can be detected. In contrast, FYDR mice yield a fluorescent signal that is specific to HDR events, and the recombination rate can be readily measured in cells from both embryonic and adult tissues. Furthermore, fluorescence makes it possible to capture in situ images of recombined cells, making it possible to discern independent lineages of recombinant cells in vivo. Our Specific Aims are to (1) evaluate the frequency of recombinant cells in multiple tissues; (2) develop methodology for quantification of recombinant pancreatic cells in situ and reveal the relative frequency of recombinant cells among two different cell types within a normal tissue for the first time; (3) measure the effects of environmental factors on recombination in vivo; and (4) reveal how specific genes (Blm and p53) affect recombination susceptibility in vivo. The broad long-term objectives of this work are to demonstrate the utility of this newly developed technology for studying

recombination in mammals, to substantially expand the capabilities of the existing system, and to elucidate environmental and genetic factors that influence a person's susceptibility to spontaneous, environmentally induced, and cancer therapy-induced DNA rearrangements.

Dissection of the Modular Structure of Cancer Signaling Systems

Meyer, Tobias, Department of Molecular Pharmacology, Stanford University 1 R21 CA1207322-01

Description: The development of human cancer is a multistep process in which future cancer cells acquire mutant alleles of proto-oncogenes, tumor-suppressor genes, and other regulatory genes. Many or most of these genes are signaling-related proteins, and we are focusing here on the design principles of signaling networks that control the cancer-related processes of proliferation, migration, and endocytosis. We will test the key questions of (1) whether these cancer-related signaling networks have a modular structure and (2) whether cancer cells have missing or added signaling modules that cannot be observed in normal cells. We have made significant advances to answer these questions by developing a method to create 2,304 in vitro Dicer generated siRNAs against a core set of human signaling proteins. Using these siRNAs, we have already discovered the function of STIM1, a Ca2+ sensor in the ER lumen that controls Ca2+ influx into cells and also acts as a tumor suppressor. We have also developed quantitative microscopy-based measurement tools to track signaling processes and cell functions. Phase 1 of the proposal will demonstrate the overall feasibility of using a microscopy-based siRNA strategy to investigate multiple cancer-related cell functions. Phase 2 will address the questions posed above using an expanded set of 6,000 siRNAs and a focus on 6 cell types, 3 nontransformed and 3 breast cancer epithelial cell lines. We will screen to identify signaling siRNAs that alter proliferation, cell migration, or endocytosis and then utilize followup studies with live cell biosensors that we developed to measure the duration of different cell cycle phases, as well as migration velocity and other kinetic parameters. We will then link genes that alter these cell functions to a subset of cancer-relevant signaling pathways using secondary siRNA screens. Based on these functional and signaling datasets, we will create a modular map of signaling systems using clustering methods. We will experimentally test the predictive power of modular maps using perturbations with pairs of effective siRNAs. We will show whether and how modularity in a signaling system can be used to predict how cell functions can be manipulated using combinations of siRNAs and learn whether and what distinguishing features exist that define modularity of signaling systems in cancer versus noncancer cells. This will likely lead to the identification of new cancer drug targets and new therapeutic strategies.

Oncogenic MicroRNAs in HPV-Infected Cervical Samples

Beaudenon, Sylvie, Ambion, Inc. 1 R43 CA116218-01 (SBIR)

Description: Cervical cancer, caused by human papillomaviruses (HPVs), is a major public health problem worldwide. About 230,000 women die of cervical cancer every year, the majority in developing countries. Although early detection via routine cytological screenings (Pap smears) and HPV testing has lowered both the incidence and mortality of cervical cancer, significant problems and barriers remain, including the low predictive value of current testing. As many as 3 million Pap smears are classified as inconclusive in the United States every year, leading to costly and invasive followup procedures and emotional stress in patients. MicroRNAs (miRNAs) are small, regulatory RNAs encoded by plant, animal, and fungal genomes that act to inhibit expression of specific target genes. Recent studies have shown that miRNAs play key roles in many cellular processes, including development and differentiation. The role of miRNAs in the development of diseases and cancers has just begun to emerge. Furthermore, recent data show that many viruses encode miRNAs that regulate both viral and cellular gene expression to establish and maintain productive infection. Work at Ambion and in Golub's laboratory has shown that miRNAs are differentially expressed in specific types of cancers, providing the first evidence that miRNAs can be used to classify human tumors and develop diagnostic assays. Our hypothesis is that miRNAs are involved in the host-cell response to HPV infection and in HPV-induced cellular transformation and that HPVs themselves encode miRNAs that are involved in these processes. We propose to investigate host-cell and viral miRNAs involved in HPV infection for the purpose of better understanding the natural history of HPV infections and the early events that lead to the onset of

cervical cancer. In Phase I, we will use an miRNA profiling system to analyze the host-cell miRNA response to HPV infection and transformation in HPV-positive cell models and cervical biopsies. We will also explore the identification and validation of HPV-encoded miRNAs and determine their relevance to HPV infection and transformation. Phase II will encompass wider evaluation of the identified cellular and viral miRNAs in clinical samples as potential biomarkers, and a diagnostic assay will be developed based on these miRNAs.

Microfluidic System for Automated Cell Toxicity Screening

Hung, P., Cellonix Corporation 1 R43 CA120619-01 (SBIR)

Description: The main objective of this proposal is to develop a microfluidic platform for cancer drug toxicity screening in cultured human cells. While it is believed that improved information on a patient's individual cancer signature can aid diagnosis and treatment, the technology available to validate this claim is currently limited. The long-term goal of this work is to commercialize a microfluidic screening platform to provide a compact, low-cost, automated screening system that can be used in the clinical setting. The specific aims of this proposal are to automate a previously developed microfluidic cell culture array and to demonstrate the feasibility and reproducibility of cancer drug toxicity screening in the microfluidic format. The design and fabrication of the addressable 8x3 unit microfluidic array will leverage expertise developed within the company related to soft lithography technology. Automation of fluidic delivery through the array will be accomplished through implementation of novel microfluidic valves controlled with an industrial pneumatic interface. Initial demonstration of cancer cell cytotoxicity will be collected on HeLa cells over 7 days of exposure to anticancer drugs such as etoposide. Cell viability as well as apoptosis kinetics (quantified by fluorescence assay) will be collected in the array and experimental robustness determined. Response and statistical uniformity will be compared to the same assay performed in a 96-well plate. The commercialization of the microfluidic platform can improve public health by providing a reliable, cost-effective instrument that can be used for personalized cancer diagnosis in the clinical setting. This technology overcomes current limitations by reducing the cost of automated cell analysis through the scalability of microfabrication and by enabling multiplexed assays on a small amount of patient tissue through reduced sample volume. A similar platform can also be adapted for molecular screening in cancer cell biology and for improved high-throughput drug discovery.

Analysis of Tumorigenic Signaling Pathways with PROTACS

Crews, Craig, Department of Molecular, Cellular, and Developmental Biology, Yale University 1 R21 CA118631-01A1

Description: A key part of determining the course of treatment for a specific cancer is the identification of the specific activated signaling pathways that are causing the malignant growth. In fact the treatment for a given cancer can be dependent upon the activated signaling pathway; for example, HER2/neu-positive vs. -negative breast cancers are treated differently. This personalized medicine approach is best exemplified by the development of the Abl tyrosine kinase inhibitor Gleevec, which has revolutionized the treatment of CML. As more drugs targeting specific signaling pathways are developed, it will be important to identify those oncogenic signaling pathways activated in a given tumor biopsy. Toward this end, our long-term goal is the development of a library of small molecules to be used as diagnostic tools for assessing primary cancerous tissue samples. We have recently developed a new technology known as PROteolysis TArgeting Chimera molecules (PROTACs) that can selectively knock down a specific protein in vivo. These cell-permeable, heterobifunctional molecules utilize the cell's own ubiquitin/proteasome protein degradation pathway to selectively destroy a target protein of our choosing. We propose to adapt this technology so that proteins required for continued tumor growth are degraded only in those cells with a particular activated tyrosine kinase pathway. In this way, it will be possible to identify those signaling pathways that are upregulated in a particular tumor cell and that are required for its growth. Toward the goal of novel tumor diagnostic technology development, in the subsequent R33 application, we propose to develop a panel of PROTACs that can be used in identifying the activated cancerous cell signaling pathways. This panel will be tested for use as a diagnostic tool for determining the best course of drug treatment.

Nanoparticles for Harvesting and Targeting Angiogenic Proteins

Ferrari, Mauro, Biomedical Engineering Center, Ohio State University 1 R21 CA122864-01

Description: This R21/R33 application has as its hypothesis that development and refinement of surface characteristics of silica chips with nanocharacteristics can enhance sensitivity of mass spectrometry (MS) detection of the low-molecular-weight angiogenic proteins present in serum and tumors that are produced at very early times of tumor development. In addition, refinement of conjugation methods of nanoporous particles will allow selective targeting of endothelial cells in vitro and tumor-associated blood vessels in vivo; in combination with refinement of loading strategies, cytotoxic agents loaded into nanoparticles can selectively destroy these vessels. Our experimental plan is based on our expertise in development and refinement of emerging nanotechnology approaches for protein capture and selective targeting and loading of silicon nanoparticles. These studies also take advantage of our experience in identification of novel proteins within the vascular endothelial growth factor (VEGF) family of proteins that are essential in the process of tumor-associated angiogenesis. To achieve the goal of developing and refining tools for detection of angiogenic proteins and for selective targeting and destruction of tumor-associated blood vessels, the following Specific Aims are proposed: (1) develop and refine silica chips with nanocharacteristics to enhance the sensitivity of LC-MS/MS identification VEGF proteins in serum and in skin tumors during skin tumor-associated angiogenesis in vivo; (2) refine conjugation of silicon nanoparticles to anti-VEGFR-2 receptor antibodies for selective targeting of endothelial cells in vitro and targeting tumor-associated blood vessels in vivo; and (3) determine the ability of silicon nanoparticles conjugated with anti-VEGFR-2 antibodies to be loaded with and to deliver the cytotoxic agent melatin for destruction of endothelial cells in vitro and for destruction of tumor-associated blood vessels in vivo. These studies will provide sensitive nanotechnology tools that are critical in defining the proteome in serum and tumors related to tumor angiogenesis that is currently unexplored. These studies may also provide strategies to selectively target tumor vessels for destruction using nanotechnology approaches.

Defining Secreted Glycan Alterations in Pancreatic Cancer

Haab, Brian, Van Andel Institute 1 R21 CA122890-01

Description: The development of methods to accurately detect early pancreatic cancer and to better differentiate benign from malignant disease could greatly improve the outcomes for pancreatic cancer patients. It is known that malignant transformation of epithelial cells of the pancreas results in alterations in the carbohydrate chains of certain proteins secreted or released by these cells. Glycosylated proteins form the basis for current biomarkers for detecting pancreatic cancer and other adenocarcinomas, and refinement of these tests is predicted to enable detection of early pancreatic cancer. Our preliminary data have shown that a novel antibody-microarray technology allows the efficient detection of glycans on distinct proteins and the identification of specific glycan structures associated with pancreatic cancer. The method uses antibody microarrays to capture specific proteins from serum samples, followed by the incubation of a glycan-binding protein (such as a lectin) to quantify specific glycans on the captured proteins. Two classes of glycoproteins, mucins and carcinoembryonic-antigen-related proteins, are particularly associated with cancer, both in altered expression patterns and in altered glycan structures on the proteins. In the R21 phase, we will determine the levels of multiple specific glycans on members of those protein classes to test the hypothesis that the measurement of specific cancer-associated glycans on specific proteins, as opposed to measuring just protein or just glycan levels, will yield improved sensitivities and specificities for cancer detection. The R33 phase of the project will expand and thoroughly test the approach. The sensitivity and specificity of detecting pancreatic cancer using measurements of glycans on mucins, CEA proteins, and proteins identified in the R33 phase will be characterized in a large set of serum samples from subjects with pancreatic cancer, benign pancreatic disease, other cancers, and no disease. We expect to characterize the value of these measurements for disease diagnostics and to gain insights into the generality and frequency of specific glycan alterations on secreted proteins. With respect to relevance to public health, the ability to more accurately diagnose cancers at earlier stages could lead to improved outcomes for many patients. This research could lead to significantly improved blood tests for the detection of cancer, as well as a powerful, generally applicable platform for studying carbohydrate alterations on multiple proteins.

Defining the Multiple Myeloma Kinome

Kerr, William, Oncology Program, H. Lee Moffitt Cancer Center and Research Institute 1 R21 CA118632-01A1

Description: Gene profiling technology has enabled analysis of the transcriptome and proteome of tumor cells, including multiple myeloma (MM). This information has provided useful information with regard to molecular mechanisms that define the enhanced survival and proliferation of MM cells. However, an equally, if not more, important goal is to define those proteins that participate in signaling pathways active in MM cells and their supporting stroma. Enzymes that phosphorylate tyrosine, serine, and threonine residues on other proteins play a major role in signaling cascades that determine cell cycle entry and survival in MM and the stromal cells that support them. In particular, knowing the signaling pathways that are active in MM cells and their supporting stroma will provide critical information for understanding MM cell survival in the BM. We have developed and are applying to purified cells a novel array-based strategy that allows the simultaneous detection of phosphorylation for 1,176 different kinase substrates. Here we propose to apply this emerging technology to the analysis of phosphorylation-based cell signaling pathways in MM and their supporting stroma. This R21/R33 Phased Innovation application will be pursued in two phases. In the R21 phase of this application, Aims 1 and 2 will validate that PepChip technology can be applied to MM cells and their stroma to reveal signaling alterations in MM cells. In Aim 3 of the R33 phase, we will use PepChip technology to identify kinome alterations within the MM patient population that are correlated with clinical parameters such as relapse and chromosomal abnormalities associated with poor prognosis. In Aim 4 we will utilize an in vivo model that supports the growth of primary patient isolates in human bone grafts to determine the effect of therapeutics on the kinome of MM cells. This study will be pursued in the following phased R21/R33 format: R21 Phase: Aim 1: Define the kinome of MM cells and normal plasma cells. Aim 2: Define the kinome of BM stroma from MM patients and normal controls. R33 Phase: Aim 3: Identify kinome alterations in MM correlated with clinical parameters of disease. Aim 4: Identify kinome alterations in MM cells in response to therapeutics in vivo.

Real-Time PCR Expression Profiling of MicroRNA

Schmittgen, Thomas College of Pharmacy, Ohio State University 1 R33 CA114304-01A2

Description: MicroRNA is a newly discovered class of endogenous, small interfering RNA. MicroRNA binds to messenger RNA and translationally represses protein levels. While over 300 microRNAs have been discovered in humans alone, their biological function, targets, expression levels, and role in disease remain largely unknown. A role between microRNA expression and carcinogenesis has been proposed. There is a lack of sensitive, high-throughput methodologies to monitor the expression of microRNAs. microRNAs are challenging molecules to quantify because the microRNA precursor exists as a stable hairpin, and the mature microRNA is only 22 nucleotides in length. We propose to evaluate sensitive and specific real-time PCR assays to quantify the expression of the mature and microRNA precursors. The microRNA expression will be analyzed in a number of important biological conditions relating to human cancer. The microRNA expression will be determined in specific sections of cancer and normal tissue isolated by laser-capture microdissection. The expression of mature and precursor microRNAs will be compared using real-time PCR and a cDNA microarray. microRNA expression will be studied in clinical samples of human pancreatic cancer. The unparalleled sensitivity and specificity of real-time PCR as applied to this new and exciting class of regulatory RNAs should propel the field into new directions not only in cancer but also in other areas of human health.

Epigenetic Targeting in Non-Hodgkin's Lymphoma

Shi, Huidong, Ellis Fischel Cancer Center, University of Missouri, Columbia 1 R21 CA123018-01

Description: Hypermethylation of promoter CpG islands plays a prominent role in cancer. In partnership with alterations in histone acetylation/methylation, this epigenetic event establishes a repressive chromatin structure that leads to silencing of key cancer-related genes. The occurrence of DNA methylation within the genome is not random, but rather patterns of methylation are generated that are gene and tumor type specific. How DNA methylation patterns are established is still poorly understood. Since various transcriptional factors

or regulators are found in association with DNA methyltransferases (DNMTs) in vivo, we hypothesize that (1) oncogenic transcription factors can recruit DNMTs to target gene promoters and define a unique epigenetic signature in tumor cells and (2) dissecting such a complex epigenetic hierarchy will identify novel molecular targets for diagnosis, prognosis, and therapeutic intervention. To test our hypothesis, we developed a high-throughput technique for genome-wide analysis of DNA methylation associated with specific proteins such as histones, transcription factors, or any DNA binding proteins. The new approach, named "ChIP-Chop-DMH," will combine both genome-wide location analysis (also known as ChIP-on-Chip) and Differential Methylation hybridization (DMH) analysis, two emerging technologies used in epigenetic research. The proposed method has distinct advantages over current protocols. First, this method directly examines the in vivo interaction of specific proteins with methylated DNA throughout the genome; second, this method may uncover novel biological properties of transcription factors; and third, this method can be applied to discover novel epigenetic biomarkers relevant to tumorigenesis. In preliminary studies, we have verified the utility of this method with methylated histone H3 at lysine 9 and lysine 4 in human cancer cells. In the R21 phase, we will continue minor refinement of the method and pursue three aims: (1) improve and optimize the ChIP-Chop-DMH method for analyzing genome-wide association of DNA methylation with histone modification; (2) utilize the proposed method to investigate the association of DNA methylation with chromatin remodeling factors; and (3) show proof of concept using the array to examine primary non-Hodgkin's lymphomas (NHLs). In this development phase, we will focus on the sensitivity, reproducibility, and accuracy of the proposed method. In the R33 phase, our goal is to utilize the technology to test biological hypotheses. We will fully implement the method and pursue these aims: (1) discover epigenetic target genes associated with known oncogenic transcription factors c-Myc and BCL6 and (2) validate the identified epigenetic targets and investigate the regulatory role of the associated oncogenic transcription factors. This systematic approach will provide a powerful tool for future mechanistic studies as well as cancer diagnosis.

MRI Contrast Agents Targeting Carbohydrate Biomarkers

Wang, Binghe, Chemistry Department, Georgia State University 1 R21 CA123329-01

Description: Malignant transformation is often associated with alteration of cell-surface carbohydrates. The expression or overexpression of certain carbohydrates, such as sialyl Lewis X (sLex), sialyl Lewis a (sLea), Lewis X (Lex), and Lewis Y (Ley), has been correlated with the development of certain cancers. These cell surface carbohydrates can be used for cell-specific identification and targeting of carcinoma cells. Recently, we have developed boronic acid-based small-molecule lectin mimics (named boronolectins) that can recognize certain carbohydrates with selectivity. The same or similar methods can be used for the preparation of lectin mimics for a wide variety of carbohydrates. The long-term goal of this project is the development of conjugates of boronolectin-MRI contrast agents as biomarker-directed cancer imaging agents. Specifically, such conjugates can be used for the delivery of MRI contrast agents based on cell-surface carbohydrate biomarkers. In the R21 phase of this application, we plan to study the feasibility of this approach by (1) synthesizing boronolectin-MRI contrast agent conjugates using a boronolectin that is known to selectively bind to sialyl Lewis X, (2) studying their ability to bind to cells with the target carbohydrate biomarkers, and (3) examining their ability to image implanted tumors in both ex vivo and in vivo models. If the R21 phase is successful, in the R33 phase we plan to expand our biological evaluation to include tumors implanted at different positions and search for other lectin mimics that can bind specifically for other important carbohydrate-based cancer biomarkers. In addition, we also plan to examine the cytotoxicity of the boronolectin-MRI contrast agent conjugates. These small-molecule-based recognition/delivery systems may have the following advantages over antibody-based systems: (1) greater stability during storage and in vivo, (2) lower propensity to elicit undesirable immune responses, (3) easier conjugation chemistry, and (4) more desirable pharmaceutical properties.

Breast Cancer Proteomic via Laser-Free Microdissection and Gemini Technologies

Balgley, Brian, Institutional Partner: Calibrant Biosystems, Inc., Academic Affiliation: Yale University 1 R41 CA122745-01 (STTR)

Description: The generation of biologically relevant proteomics data requires samples consisting of homogeneous cell populations, in which no unwanted cells of different types and/or development stages obscure the results. The problem is compounded for the analysis of tissue biopsies, since many different cell types are typically present, and small numbers of abnormal cells may lie within or adjacent to unaffected areas. While methods such as laser capture microdissection (LCM) enable the isolation of homogeneous subpopulations of cells, proteomic analysis of LCM-procured specimens is severely constrained by the very low amounts of sample generated. To avoid the limitations of established proteome techniques for analyzing protein extracts obtained from microdissection-procured tissue specimens, an effective discovery-based proteome platform has recently been developed at Calibrant. This proteome platform, called Gemini, combines a unique multidimensional separation system with customized back-end bioinformatics tools and allows ultrasensitive analysis of minute protein amounts extracted from cells captured by tissue microdissection. This project further aims to employ a novel, laser-free microdissection technique pioneered by our collaborator, Dr. Zhengping Zhuang at the National Institute of Neurological Disorders and Stroke, which is capable of providing enriched, high-quality, reproducible tissue samples. By combining Calibrant's ability to perform proteomic profiling from minute samples with the technology and expertise offered by Dr. Zhuang in tissue microdissection and tumor pathology, the proposed research represents a synergistic effort toward the evaluation and validation of a novel biomarker discovery paradigm for enabling the proteome analysis of cancer cells and their microenvironment in support of cancer research, diagnosis, and treatment. Application of the resulting biomarker discovery platform for studying the molecular mechanisms associated with breast carcinoma at the global level will be realized through a collaboration with Professor Fattaneh A. Tavassoli (Yale University School of Medicine), who will apply more than 30 years of research experience in breast cancer pathology and biology and provide access to a collection of fresh-frozen human breast cancer biopsies for biomarker discovery.

Autoantibody Profiling of Non-Small Cell Lung Cancer

Hirschowitz, Edward, Institutional Partner: 20/20 Gene Systems, Inc. Academic Affiliation: University of Kentucky 1 R41 CA118625-01A1 (STTR)

Description: Tumor markers, measured in peripheral blood, could assist in diagnosis and management of non-small cell lung cancer (NSCLC) and potentially improve historically dismal outcomes. Circulating antibodies, generated to a wide range of tumor-associated proteins, can be translated into a valuable blood test for lung cancer. Preliminary data support this hypothesis. We have successfully used phage-display, biopan enrichment techniques, and high-throughput fluorescent array screening to identify multiple known and unknown tumor-associated proteins specifically recognized by circulating tumor-associated antibodies in NSCLC patients but not in normals. A panel of phage-expressed proteins arrayed on a glass slide microarray used to measure tumor-associated antibodies in serum from a cohort of cancer patients and risk-matched controls affords predictive accuracy that exceeds that of currently available circulating NSCLC-associated protein markers. Although the fluorescent microarray system is an ideal tool for identifying proteins recognized by tumor-associated antibodies, it is not a commercial-ready platform. The intent of this application is to incorporate these markers into a layered protein array (LPA), a 96-well ELISA-type platform that has been developed for clinical diagnostics. The high-throughput format of the LPA that allows measurement of multiple antibody markers simultaneously will be central to the application and is a perfect complement to biomarker identification. The LPA will be initially constructed and tested using a panel of proteins that have already been identified. Our initial application will be early detection of lung cancer, although multiple applications in lung cancer management are rational. Data show feasibility and proof of concept that support the rationale for further development and testing of this approach. Subsequent Phase II application will evaluate an assay developed in this Phase I project for application to screening of NSCLC. Thus, the primary goal of this application is to develop a novel blood test for NSCLC that can be rapidly translated into clinical practice. Success in this project will herald similar development in other malignant diseases. With respect to relevance to public health, a blood test for lung cancer could improve the capability and cost-effectiveness of early detection as a viable strategy for reducing mortality from this disease.

FY 2007

Application of Technologies for Interactome Network Analyses of Cancer Mutations

Vidal, Marc, Department of Cancer Biology, Dana-Farber Cancer Institute 1 R33 CA132073-01

Description: The molecular mechanisms underlying cancer have been mainly studied one or a few "cancer genes" at a time. However, it is thought that combinations of mutated or aberrantly expressed tumor suppressor genes and oncogenes may be responsible for advancing cells through most steps of tumorigenesis. Many cancer-causing mutations are disrupting interactions, and these alterations are often directly related to the mechanism of pathogenesis. Thus, altered protein-protein interactions may directly point to a mechanism for the genesis of cancer. More importantly, since it is becoming increasingly clear that genes and their products interact in complex biological networks with local and global properties, it is possible that perturbations of these networks contribute to cancer formation. We propose that a further understanding of the mechanisms involved in cancer, and the development of new therapeutic strategies, can be gained by (1) studying genes and their products in the context of the molecular networks in which they function and (2) investigating how such networks are altered in tumor cells as compared with their unaffected counterparts. In addition to the information available from several drafts of the human genome sequence, genome-wide experimental strategies have been developed that will help us understand the effects of cancer mutations in the context of molecular networks: (1) protein-protein and DNA-protein interaction networks, or "interactome" networks, are being mapped at an increasing pace, producing datasets with ever-increasing quality and decreasing costs; and (2) large numbers of cancer-associated mutations are being discovered in the context of the human cancer genome project. Here we propose to develop a genome-wide application for a new technology platform that we have recently initiated to systematically study the effects of cancer-associated mutations on the physical and functional interactions mediated by the products encoded by cancer genes in the context of global interactome models. Our specific aims are to apply our experimental and computational technology platforms to (1) clone large numbers of cancer-associated missense or single amino acid change (SAC) alleles, (2) identify and characterize the interaction properties of large numbers of SAC alleles, and (3) analyze the effects of SAC alleles on the local and global properties of interactome networks.

Parallel Capillary Bioreactors for Leukocyte Transendothelial Migration Analysis

McCawley, Lisa, Medical Center, Department of Cancer Biology, Vanderbilt University 1 R21 CA126728-01A1

Description: The tumor microenvironment, and in particular tumor-associated inflammation, is a driving force of tumor progression. Immune cell (i.e., leukocyte) infiltration into sites of inflammation requires the coordinate regulation of multiple steps, including arrest on endothelium, migration through the endothelial barrier, and directed migration through connective tissue. A potential key regulator of leukocyte infiltration is a member of the matrix metalloproteinase family, MMP3. Mice that are null for MMP3 demonstrate reduced infiltration of leukocytes in a variety of model systems, and a number of matrix and non-matrix MMP substrates identified include those known to affect immune cell function. We hypothesize that MMP3, as well as other MMPs, directly enhances leukocyte extravasation during tumor progression. Direct analysis of the roles of MMPs is possible through analysis of primary cells isolated from mice with genetic ablation of individual family members. However, current technologies to assay leukocyte extravasation either do not recapitulate key physiological parameters, such as the microfluidic shear and apical-basolateral organization of endothelium, or require extensive tissue samples, which excludes their use with primary cells isolated from mice. The goal of this proposal is to exploit the expertise of our collaborative team of cancer biologists and engineers in applying soft-lithography microfabrication technology to the biological challenge of the study of tumor-associated leukocyte infiltration ex vivo. We propose a multidisciplinary approach in the development of planar and multilayer parallel capillary perfused bioreactors (PCPB) that (1) better approximate the spatial constraints and architecture of blood vasculature, (2) can provide regulated shear flow, and (3) are high-throughput in design, requiring minimal cell samples for assay conditions. The development of the planar and multilayer PCPBs for application in studying leukocyte transendothelial migration is detailed below. Completion of these aims will generate novel devices that will provide an ex vivo system that more closely

approximates physiological vasculature, facilitating novel insights into leukocyte recruitment from circulation. Specific Aim 1: To develop a planar PCPB. (A) Design and fabricate planar PCPB with a recirculating nutrient supply system. (B) Apply the planar PCPB to the assay murine-derived leukocyte attachment to endothelial monolayers. Specific Aim 2: To develop a multilayer PCPB that will support endothelial polarization into apical and basolateral surfaces. (A) Design and fabricate a multilayer PCPB that will incorporate a filter system. (B) Establish culture conditions that allow for endothelial polarization across filter of multilayer PCPB. (C) Define parameters for leukocyte attachment, rolling, and transendothelial migration in multilayer PCPB as compared with traditional transmission electron microscopy (TEM) assay.

Using New Optical Techniques to Study Cell Signaling in 3D Matrices

Keely, Patricia, Medical School, Department of Pharmacology, University of Wisconsin-Madison
1 R21 CA126635-01A1

Description: Mammary epithelial cells undergo ductal morphogenesis only in compliant 3D extracellular matrices (ECMs) (floating collagen gels or Matrigel) but not in otherwise identical matrices that are stiff (attached 3D collagen gels) or have increased collagen density. An understanding of how cancer cells interact with local tissue environments requires the ability to observe the relationship between signaling molecules, subcellular structures, and components of the ECM within a 3D environment. Few studies have investigated signaling events in 3D, and to our knowledge none have done so using live cell imaging or fluorescence resonance energy transfer (FRET) approaches. 3D imaging adds complexity to these fluorescence studies, including the inherent challenge of a 3D volume as opposed to a 2D image, problems of overlapping spectra, low signals, and light scatter. The goal of this project is to develop imaging techniques in the context of 3D culture systems to directly visualize signaling pathways relevant to breast tumor cell behavior. Our underlying hypothesis is that the Rho signaling pathway is the sensing mechanism by which the physical properties of the microenvironment are conveyed to signaling effectors within the cytoskeleton. We propose the following aims. Aim 1: Characterize endogenous signals of the 3D matrix and tissue environment within which we wish to investigate signaling events. (A) Characterize the multidimensional signals of endogenous fluorophores within cells within a 3D collagen matrix of various densities and compositions. The "fingerprint" of endogenous fluorophores (NADH, FAD, and collagen) within collagen matrices will be spatially mapped by using multiphoton laser-scanning microscopy (MPLSM) and spectral and fluorescent lifetime microscopy (SLIM and FLIM). (B) Characterize the endogenous signals in the mouse mammary gland during tumorigenesis. Endogenous signals (NADH, FAD, and collagen) will be characterized by multiphoton laser-scanning microscopy (MPLSM), SLIM, and FLIM in models of mouse mammary tumorigenesis, generating novel data regarding metabolic changes during tumorigenesis. Aim 2: Develop strategies for directly visualizing Rho/ROCK signaling pathways within 3D matrices using MPLSM and FLIM. (A) Investigate the matrix-dependent activation of Rho GTPase. Fluorescent probes coupled to Rho and a Rho binding domain will be used as FRET pairs to determine Rho activation spatially and temporally in a 3D context within collagen gels and definable microfluidic channels. (B) Apply FRET/FLIM techniques to characterize the coupling of Rho to its effector ROCK in response to collagen density. Coupling of activated Rho to ROCK and regulation of myosin-mediated contractility will be determined spatially and temporally within 3D collagen matrices.

Application of a Novel Nanotechnology for Molecular Profiling of Tumor Cellular E

Nelson, Edward, University of California, Irvine 1 R21 CA132039-01

Description: Our increasing appreciation of (1) tumor genetic and cellular heterogeneity; (2) the recent descriptions of cancer stem, endothelial progenitor, and myoepithelial cells, among others; and (3) disparate responses to treatment even for histologically similar tumors raise fundamental questions as to the relative contributions of various tumor cellular subsets to the biologic behavior of a tumor. However, technologies permitting the prospective characterization of discrete tumor cellular elements and recovery of selected viable cells from a tumor have yet to be developed. We have developed a novel nanotechnology consisting of an array of microfabricated SU8 polymer elements that permits the isolation and recovery of individual adherent cells. This advanced nanotechnology, combined with multicolor immunofluorescence and advanced confocal

microscopy, enables us to propose the application of this technology to the simultaneous identification, recovery, and evaluation of selected molecular profiles from viable primary adherent cell populations representing the various cellular elements within individual tumors. The hypothesis for these studies is that the pallet array nanotechnology will permit identification, enumeration, and recovery of individual cellular tumor elements—cancer stem, endothelial progenitor, myoepithelial, epithelial, and inflammatory cells—leading to the molecular characterization of these cellular subsets within individual tumors and will be tested by pursuing the following specific aims: (1) refine the pallet array for maximum cell capture, detection, and recovery of tumor cellular elements; (2) apply pallet array to fine needle aspirate (FNA) samples of primary breast tumors to identify and isolate individual cells from discrete tumor cellular elements; (3) establish feasibility of molecular analysis of recovered individual rare cells, e.g., single cell reverse transcriptase polymerase chain reaction (RT-PCR). These studies represent a convergence of biomedical engineering, advanced laser optics, cell biology, immunology, and clinical oncology and will drive future studies to address fundamental biological and clinical questions.

Quantitative Proteomics of Metastasis

Krizman, David Burke, Expression Pathology, Inc. 1 R43 CA132081-01 (SBIR)

Description: This Phase 1 SBIR application proposes to establish whether metastasis-associated proteins are upregulated in primary breast cancers that have shown the ability to become mobile and invade non-breast tissue and whether this phenomenon can be exploited to indicate the aggressiveness and/or metastatic potential of earlier-stage breast cancers. Predicting the clinical aggressiveness of a tumor through molecular pathology indications could have far-reaching ramifications for personalized patient management. Expression Pathology, Inc. (EPI), has preliminary mass spectrometry-based protein expression data that strongly suggest that specific proteins known to be involved in tumor cell attachment, invasion, and locomotion are upregulated in epithelium microdissected from primary tumors that had already shown the metastatic phenotype versus epithelium microdissected from primary tumors that had not metastasized at time of collection. In collaboration with the Clinical Proteomics Facility-University of Pittsburgh Hillman Cancer Center, EPI expects to apply the emerging technical capabilities of global quantitative protein expression analysis with specific emphasis on these proteins in order to firmly establish that increased expression of multiple proteins from entire families of proteins that mediate the metastatic phenotype correlates directly with the metastatic status of a primary breast tumor. This will be accomplished by utilizing Liquid Tissue® reagents to prepare soluble tissue lysates of microdissected epithelium directly from 36 formalin-fixed breast cancer tissue samples. The first set of 12 tissue samples will be from primary breast cancers in which each patient had demonstrated metastatic disease at time of presentation (Stage IV). A second, separate collection will consist of 12 primary breast cancer tissue samples from patients with tumors that showed no spread to the surrounding lymph nodes at time of presentation and who, after long-term followup, remain cancer free (Stage I). A third collection of 12 samples will be from patients whose primary tumors presented with cancer that had spread to surrounding lymph nodes at time of presentation (Stage II). Each of the 36 protein lysates will be analyzed by mass spectrometry-based global proteomic profiling and quantitative protein expression data developed using spectral count methods. Differential protein expression by mass spectrometry will be validated by immunohistochemical methods for the proteins that correlate most closely with metastatic disease. The capability to quantify multiple metastasis-associated proteins simultaneously directly in formalin-fixed breast tissue is important to this application because it allows for the study of tissue collections with extensive clinical data, which cannot be duplicated with frozen tissue collections. This fact becomes even more prominent during a Phase 2 proposal where much larger tissue collections with extensive clinical data and followup need to be interrogated. Technological capabilities co-developed and already demonstrated by collaboration between these groups in the past will form the foundation for success of this proposal. Successful achievement of the stated milestones will provide for a subsequent Phase 2 proposal to explore and develop large-scale quantitative protein expression applications of these metastasis-associated proteins to provide for clinical diagnostic and prognostic assays of formalin-fixed pathologic tissue. The goal would be to develop the ability to predict whether a primary

breast tumor had already metastasized or was likely to develop metastatic capabilities. This will be achieved by mass spectrometry quantitative proteomic technologies such as AQUA and multiple reaction monitoring through an expanded collaborative between EPI, the Clinical Proteomics Facility-University of Pittsburgh Hillman Cancer Center, and the Department of Pathology-University of Pittsburgh Hillman Cancer Center involving large collections of breast cancer tissue.

Integrated Genomic Approaches to Identify and Validate Cancer Targets

Hahn, William, Department of Medical Oncology, Dana-Farber Cancer Institute 1 R33 CA128625-01A1

Description: Most human tumors, particularly those derived from epithelial cancers, exhibit global genomic alterations that make it difficult to identify mutations that are critical for cell transformation and to define the consequences of specific cancer-associated mutations. Recent advances in technologies to identify structural changes in human cancers now make it possible to consider enumerating all of the genetic alterations harbored by a particular tumor. Despite these advances in annotating structural alterations in cancer genomes, identifying the genes targeted by specific amplification or deletion events and deciphering the function of targeted gene mutations remain a major challenge. Indeed, the parallel development of efficient methods to annotate the function of cancer-associated genes is necessary to distill validated cancer targets from this structural description of cancer genomes. This proposal focuses on the integration of newly developed high-throughput methods to functionally annotate the cancer genome. Specifically, methods to perform large-scale loss-of-function, gain-of-function, and protein-protein network analyses will be combined in a novel integrated program to identify and validate functionally important cancer genes. These studies build upon prior work by our laboratories to develop and implement genome scale RNA interference libraries, complete collections of human open reading frames (ORFs), and comprehensive protein-protein interaction maps. Although the basic tools required to perform large-scale studies are now available, the integration of such whole-genome approaches represents an entirely new endeavor that requires the further development of these nascent technologies, the fabrication of comprehensive reagents, and the creation of new ways to connect these datasets to achieve a scale beyond what has been previously performed. As such, the overarching goals of this R33 application are to apply these technologies in an integrated manner while simultaneously identifying and validating genes of particular promise for therapeutic targeting. The long-term goal of these studies is to provide a foundation for the expansion of these efforts at the genome scale.

MALDI Imaging of Cancer Signaling Signatures

Kron, Stephen, Center for Molecular Oncology, University of Chicago 1 R21 CA126764-01A1

Description: Detection of the activation of cellular kinases associated with oncogenic signaling can serve as a valuable molecular marker for cancer diagnosis and as a predictive tool for selection of therapy. In human squamous cell carcinoma of the head and neck (SCCHN), epidermal growth factor receptor (EGFR) activation is associated with therapeutic resistance, increased metastasis, and poor outcomes. Histology and tumor architecture provide complementary and critical information about cancer stage and grade. By adapting and extending emerging technologies for kinase sensor biochips, we propose to develop the capability to image the distribution of cancer signaling in tumor tissue obtained by biopsy or surgical excision. We and others have developed robust, sensitive, and specific biochip-based assays for kinase activity in cellular lysates, using immunodetection, radionuclide incorporation, or matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) analysis as a readout. We now intend to adapt these methods to create multiplexed assays whereby multiple peptides, serving as specific substrates for kinase involved in oncogenic signaling, will be reversibly linked to the surface of a biochip. This multiplexed biosensor will be exposed to thick sections of tumor biopsies by "tissue print" to allow phosphorylation of the peptides. The cellular material will be washed away, and the biochip will be interrogated by MALDI-TOF MS imaging to detect relative peptide phosphorylation, creating an image of the multiple kinase activities across the tumor section. These kinase activity images can be correlated with conventional histology and immunocytochemistry to enhance diagnosis and prognosis. To establish proof of principle in the R21 phase, methods will be developed using human SCCHN tissue culture cells and xenograft tumors grown in athymic nude mice. We will take advantage

of the activated EGFR kinase characteristic of this tumor and the availability of the specific EGFR inhibitors gefitinib and erlotinib to develop and validate our biochip sensor and imaging capabilities. We anticipate achieving sufficient sensitivity and resolution to detect SCCHN tumor islands embedded in extensive stroma.

Mass Spectrometry Methods for Probing Metabolic Dynamics in Normal and Cancer Cells

Rabinowitz, Joshua, Lewis-Sigler Institute for Integrative Genomics, Princeton University 1 R21 CA128620-01A1

Description: Many of the most clinically important chemotherapeutic agents inhibit the metabolism of tumor cells. Our overarching goal is to develop a complete and quantitative understanding of the metabolic differences between normal and cancer cells and to use this knowledge to guide the rational design of novel anticancer regimens. To achieve this goal, we are developing methods that apply state-of-the-art liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry technology to probe cellular metabolism in a dynamic, quantitative, and comprehensive manner. To date, we have succeeded in developing methods for measuring metabolite concentrations and fluxes from several microbes. Here we propose to extend these methods to enable reliable measurement of metabolite concentrations and fluxes in normal and cancer cells. Specifically, we aim to enable quantitation of the concentrations of at least 150 different known, structurally defined intracellular metabolites. We also aim to measure, using isotopic tracers, the fluxes through central carbon, lipid, amino acid, and nucleotide metabolism. We will apply the analytical technology that we develop to map major metabolic differences between normal and cancer cells and to study the dynamic response of these cells to treatment with antimetabolite anticancer drugs. The methods developed here will have long-term value for understanding the mechanism of action (and toxicity) of antimetabolite anticancer drugs, for characterizing the metabolic differences between drug-responsive and resistant cancer cells, and for suggesting new approaches to inhibiting metabolism that will specifically kill cancer cells.

Multiplexed Biomarker Panels for Early Detection of Prostate Cancer

Mathew, Anu, Meso Scale Diagnostics, LLC 1 R43 CA132852-01 (SBIR)

Description: The goal of the proposed work is the development of sensitive diagnostic serological assay panels for detection of early-stage prostate cancer. Currently, the only biomarker used in prostate cancer diagnosis is the measurement of prostate-specific antigen (PSA) in patient serum. PSA levels can be elevated by various conditions and are often not at diagnostically critical levels when cancer develops. There is a need for alternative biomarkers to more efficiently diagnose prostate cancer, particularly for early detection, when aggressive treatments would be most effective. Approaches that detect autoimmune responses to cancer-related antigens are being developed as an effective means of early cancer detection, detectible before measurable amounts of the antigens with which they react accumulate for direct antigen detection. Prostate cancer-specific immune responses are being identified by Chinnaiyan et al. using several approaches, including screening with prostate cancer-specific cDNA expression systems as well as known prostate cancer-related proteins. Great value is seen in the ability of these antigens to specifically and sensitively detect cancer when used in combination, superior to PSA-based determinations. This proposal suggests combining MSD's sensitive Multi-Array® technology with the extensive prostate cancer biomarker expertise and resources of Dr. Arul Chinnaiyan and colleagues (University of Michigan) to develop highly specific and sensitive multiplex serological screening panels for early detection of prostate cancer. Multiple distinct antigens of interest (antigen-expressing phage particles, or purified peptides) will be immobilized in MSD Multi-Array® panels, which can accommodate 1-25 assays per well of a 96-well plate. These panels will be optimized for ability to detect specific humoral responses in prostate cancer patient samples, allowing multiple simultaneous determinations per well. MSD multiplex panels for serum-based measurements have shown sensitivities as low as 0.1 pg/mL, 3-5 orders of magnitude dynamic range, rapid throughput, and minimal sample usage (25 microliters per well), factors which would be critical in developing successful serological screening panels for the proposed study. The proposed academic/industry collaboration addresses NCI goals of translational medicine by advancing potential biomarkers from discovery toward clinical applications using a versatile and robust assay platform, as well as the IMAT goal of evaluating technologies that are ready for initial clinical or laboratory application in cancer research.

Microfluidic Platform for Cancer Cell Culture and Analysis

Lee, Philip, Cellasic Corporation 2 R44 CA120619-02A1 (SBIR)

Description: This Phase II SBIR proposes to develop a microfluidic cell culture and analysis platform for the in vitro screening of cancer cells. The end result of this research will be to deliver a fully functional system (automated instrument and disposable microfluidics) that can be used in both research and pharmaceutical laboratories. This will be validated and applied to the research that is under way at our collaborating institute for the purpose of profiling the Raf-MEK-ERK pathway in a panel of approximately 60 breast cancer cell lines for improved prediction of therapeutic response. The microfluidic platform will provide key advantages over the current cell-based screening technology (96-well plate based), including (1) improved handling of small cell samples (micoliters per array), (2) the ability to design more relevant microenvironments for phenotype analysis, (3) enabling multiplexed continuous flow experimentation, and (4) a 10- to 100-fold reduction of time and cost for cell culture automation. In addition, the platform is designed so that application-specific microfluidic arrays can be utilized with a single system, increasing the flexibility and impact of the technology. The first major aim of this project will be to engineer an automated microfluidic screening platform. The main tasks are to optimize the design of the Phase I prototype, scale up to a 384-well format, and refine the control system for high-throughput operation. Three key innovations developed in our previous work will be further expanded to complete this aim: (1) the design of microfluidic networks and perfusion barriers to better approximate in vivo culture conditions, (2) the use of a pneumatic pressure-driven manifold for multiplexed, non-wetted pumping of nanoliter volumes, and (3) the fabrication process that enables formatting the microfluidic arrays to SBS standards, making it compatible with current 96- and 384-well robotic screening instrumentation. The second major aim will be to apply this system to the cancer cell screening program at our collaborating institute. This will address areas in which microfluidic technology can offer enabling benefits that are not possible with existing tools. Specific applications include (1) flow-based drug exposure, (2) cell invasion assay, (3) 3D ECM culture, (4) medium conditioning by stromal cells, and (5) integration with RNAi methods.

Transcription Factor Reporter Technology

Bogdanov, Alexei, Department of Radiology, University of Massachusetts Medical School, Worcester
1 R33 CA134385-01

Description: Molecular dissection of abnormal regulation of cancer gene expression has revealed many potential targets for cancer therapy. Those targets include the components of normal as well as abnormal transcription machinery. Proteins involved in regulation of transcription in cancer will attain high priority due to the convergence of many signal transduction pathways at the transcriptional level. New molecular therapies directed to transcriptional targets, including siRNA technology, have significant advantages over traditional therapies due to a precision of their interference with target gene expression. Although rapid progress in molecular genetics and medicinal chemistry delivers new "attenuators" of gene expression, there is a critical need to develop technologies that enable early and noninvasive assessment of cancer response to these therapies. In particular, enabling imaging technologies that report directly on gene transcription in cancer cells are critically important for both cancer phenotyping and staging, as well as for evaluating new therapies. Optical imaging in the near-infrared range of fluorescence, combined with the use of enzyme-specific self-quenched probes, has emerged as a novel technology of live cancer cell screening. We previously devised a family of fluorescent probes based on synthetic biocompatible carriers of fluorochromes that report on hydrolytic activity in tumor-bearing animals. Recently, we developed new chemistry for generating asymmetrical as well as symmetrical oligonucleotide molecular reporter probes (ODMR) designed to sense interactions with pleiotropic and evolutionally conserved components of transcriptional factor NF-κB. NF-κB plays one of the key roles in tumor progression by regulating expression of cell adhesion and antiapoptotic and cytokine-responsive genes in cancer and stromal cells in tumors. We recently tested novel synthetic approaches for introducing hydrophilic, non-interfering linkers into κB-box sequences for covalent binding of fluorochromes to these probes to any internucleoside phosphate. We propose to develop ODMR technology that will be essential for the further advancement of in vivo imaging of cancer-related target transcription activators.

Sequencing Chr. 6 in Melanoma Patients

Edwards, Jeremy, Molecular Genetics and Microbiology, University of New Mexico 1 R21 CA125397-01A2

Description: Herein we propose to further develop and utilize an ultra-high-throughput DNA sequencing technology to generate large amounts of genome sequences to identify and characterize genomic variation in a population of persons diagnosed with invasive melanoma. Specifically, we will ultimately sequence chromosome 6 from 10 carefully chosen individuals. As a quantitative milestone, we will report the sequence for chromosome 6 to at least 85 percent completion for these 10 people at better than 99.999 percent assembled accuracy. In a future R33 proposal, we will collect sequences for 290 additional people. In the end, we will have a robust platform for generating a high-throughput nucleic acid sequence and converting these data to genetic markers that identify persons predisposed to progressive malignant melanoma. Current progress toward our goals is at an advanced stage. We are able to routinely sequence bacterial genomes, and we have the potential to generate enough data to rapidly sequence a single human chromosome >20x coverage in less than a month. Therefore, we feel that much of the technical risk has been reduced by our previous work, and there are substantial rewards to be gained by pursuing the goals described here.

Microfluidic System for High-Throughput Evaluation of T Cell Functionality With H

Lu, Hang, Georgia Institute of Technology 1 R21 CA134299-01

Description: Adoptive transfer of T cells is a promising clinical cancer therapy that relies on enhancing the adaptive immune response to target tumor cells in vivo. Widespread application of this therapy, however, has been hindered by the necessary expansion of large populations of T cells for each patient (often selected for tumor antigen specificity) and loss of functionality of the T cells post-transfer. Our long-term objective is to understand how T cell activation is dampened in vivo by the tumor milieu and to be able to evaluate the responsiveness of ex vivo expanded T cells accurately for cancer therapy. Microfluidic chips are ideal for high-throughput parallel experimentation and automation. In addition, microfluidics also provides the relevant length scales (~microns) and unique physical phenomena (e.g., laminar flow) to handle cells. The type of multiplex data that we can obtain from this technology will enable quantitative modeling of T cell activation and better understanding and characterization of anergy. The objective of this R21 project is to engineer a multiplex microfluidic assay to quantify T cell activation on a small population of cells with high temporal resolution. The hypothesis is that capturing the early dynamics of T cell activation of ex vivo expanded clones would improve upon current measures of T cell functionality. The first component of this project is to develop the high-throughput microfluidic system for multiple time-point stimulation and lysis of cells; in parallel, we are to develop biochemical assays to characterize the performance of the system and the cell state. The second component is to perform in vitro characterization of ex vivo expanded T cells for distinguishing anergic versus responsive behavior. The approach is innovative because the technology developed here dramatically increases the capabilities and throughput of existing assays in evaluating T cells for adoptive transfer. Furthermore, this work proposes and tests a new paradigm in T cell evaluation by multiplex quantitative means. The proposed research is significant because it is expected to expand the toolbox of cancer therapy and possibly other related quantitative biosciences and medical technologies.

Zinc Finger Recombinases for Endogenous Genome Tailoring

Barbas, Carlos, Molecular Biology and Chemistry, Scripps Research Institute 1 R21 CA126664-01A2

Description: This proposal seeks to develop a strategy of genomic recombination that is suitable for applications in both cancer research and therapy and is a revised resubmission of RA-CA-126664 responsive to RFA-CA-07-002. At present, there is no method for targeted and site-specific recombination of the endogenous human genome. Such "genome surgery" would enable the genetic dissection of cancer biology as well as give rise to a new approach to gene therapy. The rationale for the proposed work is that RecZFs, with their capacity for efficiently excising, inverting, or integrating large segments of DNA in many sequence contexts, are a unique and powerful tool for both introducing and repairing genetic defects related to cancer. This strategy contrasts with current gene therapies, which provide only transient gene expression or risk nonspecific genomic integration of exogenous DNA. The proposed research focuses on the TP53 locus, a crucial tumor suppressor, which is functionally inactivated in many different kinds of human cancer. The overall objective of this research proposal is to develop tools for efficient and selective integration into any genomic site, using the TP53 locus as a model target. The central hypothesis is that RecZFs, constructed from novel zinc finger proteins and evolved catalytic domains, will be able to perform this task. As powerful tools for genetic manipulation, we anticipate that RecZFs could facilitate the creation of novel animal models of cancer, the application of gene therapy, and the elucidation of cancer genetics.

Multiplexed Assays on the BioCD for Acute Lymphocytic Leukemia

Nolte, David, Purdue University 1 R21 CA125336-01A2

Description: The BioCD is an emerging label-free assay technology with potential for high multiplexing and high throughput to screen for many analytes across many samples simultaneously. The basis of the BioCD technology is rapid optical interferometric scanning. Interferometry is the most sensitive and quantitative means of direct optical detection. It is faster than fluorescence, with better signal-to-noise ratio, and it requires no labels, which is essential for multiple analyte detection. The broad, long-term objectives of this proposal are to apply the BioCD for the first time to the prognosis of cancer. The goal is to assay multiple biomarkers across a large cohort of 300 patients, at a level previously inaccessible to immunohistological arrays, to predict patient outcome in response to chemotherapy. The specific aims of this proposal are to establish standard response curves for a set of biomarkers relevant for predicting chemotherapy outcome for patients with acute lymphocytic leukemia (ALL) and to conduct a comparative study between assays performed on the BioCD and assays performed previously on a limited number of tissue microarrays. This is followed by the aim to scale up the capacity of the BioCD to screen for an expanded biomarker set across a set of samples already collected from the cohort of 300 patients with acute lymphocytic leukemia. The research design and the methods for achieving the stated goals rely on high-capacity automated protein spotters and high-speed laser interferometric readers. Standard concentration curves will be established by using commercially available antigens in tissue lysates, followed by measurements of marker concentrations in healthy tissue lysate. These measurements set the gold standard against which the expanded marker set measured across the large patient cohort will be compared. The relevance of this research to public health is the expansion of the marker and sample base to establish stronger clinical confirmation of the prognostic value of inactivation of a molecular pathway in patients with standard-risk acute lymphocytic leukemia and to establish the BioCD as a novel high-capacity resource for diagnostic and prognostic applications for cancer.

Innovations in Cancer Sample Preparation

Objectives and Scope

Sample preparation methods and technologies may be developed for sample collection, processing, isolation, storage, purification, preservation, and, in the case of stored tissues, reversal of adverse events resulting from storage and preservation. Methods may be for preparation of molecules, fluids, tissues, or any other samples necessary for cancer research. Researchers may propose to develop methods to isolate cells or subcellular components, such as classes of molecules, organelles, or subcellular structures. They may propose to isolate specific classes of molecules, such as membrane-bound proteins. They may also propose studies to determine the effects of collecting, processing, and storing molecular components of interest in stored specimens. The goal is to develop products and methodologies that maximize the quality and utility of samples for research and, in the case of human specimens, maximize the quality of the samples for research and clinical needs without compromising patient care.

Sample preparation methods may impact the results or interpretation of biological studies. Investigators may apply different methods of sample preparation using the same measurement technology. In many cases of measuring biological response, no "gold standard" exists by which to compare research results obtained from the different sample preparation methods. There is a need for methods to assess the quality of samples prepared using different methodologies. This RFA will support methods to assess sample quality and studies that elucidate the criteria needed to judge sample quality under different conditions. This RFA will also support the development of technologies to make these assessments, such as the development of sample reference materials that can be used to calibrate the effectiveness of new fixatives or new detection methodologies.

It is expected that many investigators who developed successful cancer sample preparation techniques under previous IMAT initiatives or under the new RFA (Innovative Technologies for Molecular Analysis of Cancer, RFA CA-06-002) (http://grants.nih.gov/grants/guide/rfa¬files/RFA-CA-06-002.html) will propose projects for this RFA. However, this RFA is not limited to techniques developed under the IMAT program. Investigators are encouraged to use any sample preparation methodologies or techniques relevant to cancer.

For all projects proposed, it will be important to substantiate the ultimate value of the innovation for analyzing samples, optimizing analysis, and/or evaluating sample quality for the purpose of research and eventually clinical applications. Also of importance is the potential for ultimately transferring knowledge, technologies, and/or methodologies to other laboratories or the clinic. In the case of technologies intended for use on clinical specimens or in patients, applications from or collaborations with investigators involved in the clinical research of cancer are encouraged.

FY 2005

Tissue Print Micropeels for Molecular Profiling Cancer

Gaston, Sandra M., Division of Urology, Department of Surgery, Beth Israel Deaconess Medical Center 1 R21 CA112220-01

Description: Molecular profiling has emerged as an important strategy for identifying marker "signatures" associated with the biological changes that characterize specific cancers. To realize the full potential of the wealth of new biomarker information, it is essential to develop strategies for profiling human tissue and tumor specimens that are workable in a clinical setting. Clinical specimens are heterogeneous, and tissue heterogeneity is one of the major sources of complexity that must be addressed in the application of molecular profiling to the analysis of human cancers. We have developed a set of novel "tissue print" techniques that allow us to profile the molecular markers over extended areas of human tissue and tumor samples without damaging the specimen. We first applied our new tissue print techniques in the profiling of protein markers associated with capsular invasion in radical prostatectomy specimens. More recently, we have discovered that, during tissue print collection, we can peel a layer of cells off the specimen and that this process does not cause detectable tissue damage (as determined by surgical pathologists), and thus does not interfere with routine clinical surgical pathology. We have also shown that the cells collected in the tissue print "micropeel" are adequate for PCR and quantitative rt-PCR analysis, allowing us to score multiple molecular markers and assemble the results in "tiling patterns" corresponding to the specimen surface. In the project outlined in this proposal, we will work closely with the research and development team at Qiagen Inc. to optimize the yield of mRNA and DNA from our tissue print micropeels collected from human prostate and breast tissue/tumor specimens. We will then develop a proof-of-principle pilot application of the tissue-print micropeel sampling technique for prostate needle biopsies, one of the classes of specimens that must be conserved intact for clinical diagnosis. Our long-term goal is to utilize tissue print techniques in the clinical setting to simplify the process of obtaining an adequate representation of human cancers in biopsies and surgical specimens and to develop protocols for this tissue sampling platform to support both proteomic analysis and PCR-based DNA and mRNA profiling techniques. In addition to facilitating basic and translational research, the tissue-printing platform can also be utilized as a tool for dedicated clinical applications to provide "molecular sections" of extended areas of the specimen when the marker profile is itself of potential diagnostic importance.

Automated Cell Preparation in Tubes for 3D Microscopy

Meldrum, Deirdre R., Department of Electrical Engineering, University of Washington 1 R21 CA112149-01

Description: 3D microscopy represents a powerful new cell analysis tool for early detection and diagnosis of cancer, but its future use may be limited because methods for preparation of samples are cumbersome, inefficient, labor intensive, and generally imprecise. Current methods for cytological sample collection are manual and distributed in nature through various physicians' office laboratories and local hospitals, with the actual analysis being centralized at regional clinical laboratories. Among the cytological specimens are sputum, gynecological and colorectal scrapes, fine-needle aspirates, and urinary tract and gastrointestinal samples. We propose the development of a new automated system that will transform these difficult and messy clinical specimens into an optimal format for 3D microscopy morphological and molecular analysis. The model and method we propose comprises three sequential steps. First, at the distributed site, an automated sample processor dissociates and fixes cells and debris for shipment in an automation compatible canister. Second, after shipment to a centralized clinical laboratory, the specimen canisters are loaded into an automatic processor that performs cleanup (debris removal), specimen/assay specific staining (and counterstaining), and finally embedding of cells of interest in glass microcapillary tubes (about 50 mu m ID), with cells being spaced at regular intervals (about 200 mu m) within a tube. This preparation format is uniquely suited for integration with multiple 3D imaging platforms for true 3D volumetric assessment of cell morphology and molecular probe and/or stain density distribution. The proposed system also enables use of cytometric flow sorting for enrichment of cells of interest at an intermediate stage of the sample preparation process. The potential impact to improved human health through rapid diagnostic screening will be illustrated using a high-impact

emerging technology, optical tomography. In summary, the aim of the proposed project is to develop, design, and build a complete sample processing system that automates the process of sample cleanup, assay-specific staining, and mounting of cells into glass microcapillary tubes and a tube positioning and rotation scanner mechanism for 3D microscopy analysis of cell morphology for the early detection of cancer.

Development of an Automated Frozen Sample Aliquotter

Larson, Dale N., Department of Biological Chemistry and Molecular Pharmacology, Harvard University Medical School 1 R21 CA114167-01

Description: Biorepositories are a valuable resource in translational research for cancer, and there are many such repositories in both academic and industrial settings. While these repositories are valuable, they are not immune from cost constraints, and the approach to storing biological specimens (e.g., serum and plasma) involves a fundamental cost tradeoff between storing the samples in a larger number of vials each with volumes (100 fl to 400 fl) suitable for assaying or storing the samples in larger volumes (2 mL or 4 mL) to save freezer space. The first approach avoids downstream aliquotting and has only one freeze/thaw cycle but requires labor to aliquot the fresh sample and is volumetrically inefficient. The second approach requires aliquotting when the samples are requested, and as a result the sample experiences a freeze/thaw cycle when it is processed for a study but is volumetrically efficient. A hybrid approach is also pursued where fresh samples are initially stored in a volumetrically efficient format until they are requested for a study and then returned to storage in the volumetrically inefficient format to avoid subsequent freeze/thaw cycles. This project will develop an automated instrument that will offer a new approach, combining the volumetrically efficient storage with the single freeze/thaw cycle. This instrument will extract aliquots from frozen samples without thawing the samples. Not only will this approach reduce the cost of operating these repositories while eliminating the second freeze/thaw cycle associated with the hybrid approach, but because it is automated, it also will increase the throughput in processing samples, reducing the 6½ weeks to 1 week for a 1,000-sample study, which is typical for the Nurses' Health Study. This team has demonstrated the ability to (1) maintain the sample at -70 °C during processing; (2) extract aliquots from normal saline frozen at -80 °C, and (3) manage frost buildup during the procedure. The R21 project and the milestones have been developed to address the key technical risks associated with this instrument, and the R33 project will deliver a prototype instrument that is suitable for use by the Nurses' Health Study to process samples for their collaborators. The design at this point will be documented well enough to enable its production in our laboratory in small quantities for use in other biorepositories.

OmniPlex™-Amplified DNA and RNA Samples From Fixed Tissue

Kurihara, Takao, Rubicon Genomics, Inc. 1 R43 CA114128-01 (SBIR)

Description: A proprietary OmniPlex™ technology will be optimized to prepare amplified DNA and RNA samples from formalin-fixed, paraffin-embedded (FFPE) tissues. These OmniPlex™ products will have predictable performance in downstream applications and allow meaningful genome-wide genetic and gene expression analyses of formalin-fixed tumor specimens for cancer research and diagnostics. These analyses are currently challenging, because no technology can effectively amplify FFPE DNA and RNA samples of variable quality and produce amplified samples with predictable, standardized quality. In the basic OmniPlex™ process, high-quality DNA and RNA samples from fresh tissues and cultured cells are amplified with robust and reproducible efficiencies, but FFPE samples are frequently damaged and/or degraded and amplify with unpredictable and highly variable efficiencies. In Phase I the basic OmniPlex™ process will be modified in order to produce more robust and reproducible FFPE sample amplification efficiencies, which are required for producing amplified products with predictable, standardized quality. Specific Aim 1 is to identify a real-time quantitative PCR assay that will normalize input FFPE DNA sample amounts better than mass measurements. Specific Aim 2 is to improve the conversion of FFPE samples to amplifiable OmniPlex™ libraries by modifying reagent and incubation conditions. Finally, Specific Aim 3 is to use in-house quantitative PCR assays to develop quality parameters that predict amplified FFPE sample performance in genetic and gene expression studies. Successful Phase I project results will lead to Phase II studies that test the robustness of the improved process on a large set of normal and tumor samples, uniquely allow DNA and RNA amplification from the same samples,

and demonstrate the utility of amplified FFPE samples for cancer research on major commercial genetic and gene expression platforms. The potential commercial applications of this research will be OmniPlex™ FFPE amplification kits and amplification service projects.

Ultrarapid Methods for Streamlined Tissue-to-RT-PCR

Latham, Gary, Ambion, Inc. 1 R44 CA097482-02A1 (SBIR)

Description: This Phase II proposal aims to simplify, expedite, and stabilize the recovery of high-quality RNA from tissue samples. In Phase I, we demonstrated the feasibility of a hands-off, closed-tube tissue disruption method termed "MELT" (multi-enzymatic liquefaction of tissue). MELT enlists potent catabolic enzymes to liquefy tissue within minutes without invasive mechanical force. High yields of intact RNA are obtained after MELT. Importantly, MELT enzymes destroy cellular RNases and stabilize RNA in tissue lysates for up to 5 days at ambient temperatures. Additionally, MELT is compatible with freshly harvested, flash-frozen, or RNAlater®-treated tissues, both mouse and human, including tumor specimens. Taken together, these advances promise faster, simpler, safer, and more robust methods for stabilizing and quantifying gene expression in tissues through innovation in RNA stability, closed-tube tissue disruption, and rapid single-tube sample preparation. In Phase II we will integrate continuing MELT enhancements with new ways to facilitate RNA processing. First, we will accelerate MELT tissue digestions and maximize the quality of the resulting RNA. Second, we will link MELT improvements with novel magnetic beads that can enable the purification of DNA-free RNA in as little as 20 minutes. This method will secure the recovery of large amounts of RNA for analysis by any expression profiling method, including microarrays. Last, we will enable an ultrarapid RNA sample preparation strategy specifically suited for qRT-PCR ("Tissue-to-RT-PCR") that skips RNA isolation altogether. Using novel approaches for tissue disruption, RNase control, DNA removal, and the management of RT-PCR inhibition, we will enable Tissue-to-RT-PCR in less than 10 minutes, with all of the steps but the RT-PCR reaction itself occurring in the same tube. Success in these objectives will result in easy-to-use products that offer improved RNA yields, greater sample throughput, more ready automation, and reduced variability, contamination, and biohazard risk compared to current methods. The beneficiaries of MELT technology will include life science researchers and clinical diagnostic laboratories, where emerging RNA biomarkers can be combined with simpler sample preparation methods to hasten the adoption of molecular diagnostic procedures.

Preparation of Cancer Tissues for MS Imaging of Proteins

Chaurand, Pierre, Biochemistry Department, Vanderbilt University 1 R33 CA116123-01

Description: Direct tissue profiling and imaging mass spectrometry (MS) provides a molecular assessment of numerous expressed proteins within a tissue sample. MALDI MS (matrix-assisted laser desorption ionization) analysis of thin tissue sections results in the visualization of 500-1,000 individual protein signals in the molecular weight range from 2,000 to over 200,000. These signals directly correlate with protein distribution within a specific region of the tissue sample. The systematic investigation of the section allows the construction of ion density maps, or specific molecular images, for virtually every signal detected in the analysis. Ultimately, hundreds of images, each at a specific molecular weight, may be obtained. To date, profiling and imaging MS has been applied to multiple diseased tissues, including human non-small cell lung tumors, gliomas, and breast tumors. Interrogation of the resulting complex MS datasets using modern biocomputational tools has resulted in identification of both disease-state and patient-prognosis-specific protein patterns. These studies suggest that such proteomic information will become more and more important in assessing disease progression, prognosis, and drug efficacy. Molecular histology has been known for some time, and its value is clear in the field of pathology. Imaging MS brings a new dimension of molecular information that specifically focuses on the disease phenotype. One important aspect of the MALDI MS imaging technology is sample preparation and processing. We propose here to further optimize the existing methodologies to maximize the information recovered from the MS analysis of fresh-frozen sections and develop and validate new approaches to investigating solvent-fixed biopsies. Next, we propose to further develop and optimize methodologies to measure pharmaceutical compounds by MS in tissue sections. We also propose to further develop and validate protocols for molecular analysis by MS of cancer cells in fine-needle aspirates. Finally, we propose to automate some key aspects of these methodologies.

Genome Amplification Tolerant to Sample Degradation

Makrigiorgos, Mike, Radiation Oncology Department, Dana-Farber Cancer Institute 1 R21 CA111994-01A1

Description: Genomic, epigenetic, and gene expression analysis from archived formalin-fixed, paraffin-embedded (FFPE) tissue samples with known clinical outcomes provides a unique opportunity for extraction of genetic information leading to improved cancer diagnosis, prognosis, and therapy. However, extensive genotyping or microarray profiling on homogeneous cell populations within these samples often requires whole-genome/mRNA amplification prior to screening. Major hurdles to this process are the introduction of amplification bias and the inhibitory effects of formalin fixation on DNA/RNA amplification. We have developed (RCA-RCA), a novel method based on isothermal rolling-circle amplification, that overcomes the limitations and promises to provide the needed link between obtaining a minute biopsy from partially degraded FFPE samples and genotyping or microarray screening. RCA-RCA enables whole-genome/mRNA amplification that can be adjusted to the degree of FFPE sample degradation, as this is assessed via real-time PCR. Thereby, RCA-RCA enables retrieval of the maximal possible amount of information from the degraded sample. In the revised application, apart from adopting the Study Section's recommendations, a further enhancement of RCA-RCA is included, mRCA-RCA. mRCA-RCA amplifies DNA while retaining epigenetic modifications on a genome-wide basis ("whole methylome amplification"), thereby allowing highly expanded detection of methylation in fresh or FFPE samples. The R21 phase will examine the maximal capabilities of the technology and establish criteria for adjusting RCA-RCA to conform to the condition of the specific FFPE sample. The R33 phase will develop the technology for obtaining minute cancer biopsies from FFPE samples, assessing sample quality, and amplifying the whole genome/methylome (DNA) or transcriptome (RNA) without introducing amplification bias. Subsequently it will establish criteria and will validate the utility of the amplified material as input for the most frequently used molecular assays (mutation/SNP detection, microsatellite instability/LOH, array-CGH, expression profiling, and methylation detection). By removing problems associated with sample degradation and biases associated with amplification, this project will enable application of the newest technologies to the analysis of minute biopsies from archived tissue with known outcomes, thereby accelerating the process of candidate gene discovery.

Magnetic Nanobeads for Cancer Cell Selection

Rampersaud, Arfaan, Columbus Nanoworks, Inc. 1 R43 CA116048-01 (SBIR)

Description: The aim of this proposal is to develop high-resolution magnetic nanoparticles for use in Quadruple Magnetic Sorting (QMS). QMS is a flow-through immunomagnetic separation system that can provide sensitive enrichment of circulating cancer cells in blood as well as other biological fluids. Optimal cellular separation by QMS requires immunomagnetic particles having high magnetic susceptibility, narrow particle size distribution, and high-density attachment sites for antibodies. Current commercial immunomagnetic beads are either too large, lack size uniformity, or have magnetic susceptibility that is too low. We will make the necessary improvements in the magnetic immunobeads using recent advances in nanoparticle technology and evaluate their performance by enriching ovarian cancer cells from a complex population of cultured cells. In Specific Aim 1, we will synthesize and physically characterize monodisperse paramagnetic nanoparticles using sol-gel methods to encapsulate Fe nanocrystals. These nanoparticles will have uniform size distribution and shape and possess a high weight percentage of iron (Milestone 1). In Specific Aim 2, we will use siloxane treatments and heterobifunctional coupling agents to create high-density attachment sites for antibodies and streptavidin (Milestone 2). The resulting particles will be analyzed for their particle field interaction parameter values, a major determinant of the effectiveness of magnetic nanoparticles in cellular separation (Milestone 3). Finally, in Specific Aim 3, we will evaluate our magnetic nanoparticles for immunomagnetic detection and separation of ovarian tumor cells by QMS. The human ovarian tetracarcinoma PA-1 tumor cells will be diluted into whole blood and subjected to QMS using antibodies against TAG-72, a surface-expressed protein present on ovarian cancer cells. Demonstrating the detection of tumor cells in whole blood cells will be taken as the proof of principle for this project.

FY 2006

Recovery of RNA From Formalin-Fixed Tissues

O'Leary, Timothy, American Registry of Pathology, Inc. 1 R21 CA118477-01

Description: High-throughput molecular biologic and proteomic methods provide several promising approaches for relating genetic changes, such as mutation or altered gene expression, to metastasis, to treatment outcomes, and to survival. In cancers where the interval between initial diagnosis and treatment and the appearance of metastases is long, clinical correlations would be more readily obtained if formalin-fixed paraffin-embedded (FFPE) tissues could be used instead of fresh or frozen specimens. Large-scale multiplex techniques, such as serial analysis of gene expression (SAGE), and gene chip methods yield experimental results that are somewhat different for FFPE tissue and unfixed tissue. The long-term goal of our research program is to use high-throughput molecular biologic screening methods to identify the molecular and genetic bases of cancer origins and behavior. The objective of this application is to identify the formaldehyde-induced chemical modifications that occur in nucleic acids during histologic tissue processing and to develop methods to reverse these modifications. Our central hypothesis is that formaldehyde adducts and cross-links formed during tissue processing can be sequentially reversed by a series of heating and dialysis steps, carried out under appropriate salvation conditions. We formulated this hypothesis on the basis of preliminary data showing that the reversal of formaldehyde-induced chemical changes in proteins and nucleic acids is relatively facile in aqueous solutions, but less so following dehydration in the presence of organic solvents. The rationale for these studies is that their successful completion will provide a foundation for applying high-throughput screening methods to FFPE tissues. This will lead to improved practical interventions for the diagnosis, evaluation, treatment, and prevention of cancer and facilitate the development of therapeutic agents. Our studies are innovative in that we have pioneered a novel model system (tissue surrogates) ideally suited to identify the formaldehyde-induced modifications to proteins and nucleic acids that occur during tissue processing. At the completion of this project, it is our expectation to have established a comprehensive understanding of the formaldehyde-induced chemical modifications to mRNA that occur during tissue histology and methods for optimally reversing these modifications. This knowledge should result in an ability to carry out genomic analysis on FFPE tissue, significantly expanding our capability to conduct genomic research and opening important new areas to practical investigation.

Develop and Validate an Ultrasound Tissue Preservation Device

Zhu, Z., Armed Forces Institute of Pathology 1 R43 CA115041-01A2 (SBIR)

Description: The primary goal of Bio-Quick is to provide a vehicle to transform the innovative biotechnology available in research laboratories from the Armed Forces Institute of Pathology into marketable and profitable medical instruments that can greatly benefit the health care, food market safety control, and advancements in the medical community. Formalin fixation and paraffin embedding (FFPE) is a time-consuming but standard tissue preservation and processing method used in over 90 percent of cases in hospitals and clinical settings for routine histology diagnosis. Our proposed project is to design and develop an ultrasound-facilitated processor (DTP) for rapid tissue fixation and processing for histology diagnosis and any further molecular study if necessary. The implementation of the technique will allow a significant reduction in processing time from at least 24 hours by conventional FFPE to less than 1 hour. We also need funding to support collaboration with outside and independent researchers to provide objective evaluation of the technique. The Specific Aims of this SBIR phase I project are (1) development of a commercialized intensity adjustable benchtop fixer/processor for rapid FF and PE and (2) evaluation and validation of the DTP method in comparison to conventional FFPE method based on preservation of morphological details and molecular analyses. During the past 7 years, we have compared the DTP method with the conventional FFPE method on over 100 human tissue specimens of 14 tissue types. Our preliminary data have demonstrated that compared to conventional FFPE, ultrasound (US)-facilitated FFPE not only significantly reduces the total fixation/processing time from over 24 hours to within 1 hour but also preserves similar or better tissue morphology, with much improved protein antigen properties and mRNA integrity. As a result of improved preservation of macromolecules, antigen retrieval treatment prior to IHC staining may be reduced; much reduced

(20X or more) antibody concentration and shortened IHC reaction time are used. Long-term stability of tissue morphology and mRNA integrity in USFFPE tissues is slightly better than that in conventional FFPE tissues.

Ultrasound-Accelerated Tissue Formalin Fixation and Paraffin Embedding

Chu, Wei-Sing, American Registry of Pathology, Inc. 2 R33 CA091166-03A1

Description: The first crucial step in cancer management is to ensure timely and accurate pathological diagnoses. Formalin fixation and paraffin embedding (FFPE) has been a standard tissue preservation method employed in over 90 percent of cases for clinical histology diagnosis. Though it provides superior morphology and easy long-term storage of clinical specimens, FFPE is time-consuming and does not fully support current molecular analyses. The long-term goal of our research is to apply modern techniques to medical practice and pathological diagnosis to effectively fight cancers and other diseases. Our proposed project is to further develop the ultrasound (US)-accelerated tissue preservation (UTP) technology for multiple tissues and to study the mechanism of US-facilitated FF. Our specific aims are to (1) develop a multitissue preservation processor with an optional real-time digital system to monitor and standardize tissue fixation level; (2) validate the UTP techniques by performing more statistical assessments on histopathology, macromolecule integrity, and their long-term stability; and (3) conduct mechanism studies to elucidate the effects of FF with and without US on the formation of cross-linking, enzymatic activity, and protein conformational changes. We have demonstrated that in comparison to conventional FFPE, UTP provides similar preservation in tissue morphology with similar long-term storage stability, improved preservation of protein structure, antigen properties, and mRNA integrity. UTP allows easy general molecular profiling and analyses based on extracts from UTP-fixed tissues. UTP also provides a good opportunity to control and monitor fixation level by adjusting the time and strength of the ultrasound. We hypothesize that US-facilitated FF will greatly accelerate formaldehyde-induced macromolecule cross-linking in tissues and "freeze" macromolecules and their conformation due to accelerated fixation reactions. Since tissue preservation is still a standard and general requirement before histology diagnosis, the innovation should have great impact in economy and public health.

Novel Method for Isolating Actively Translated mRNAs

Ju, Jingfang, Cancer Genomics Laboratory, Mitchell Cancer Institute, University of South Alabama 1 R21 CA114043-01

Description: Transcriptional regulation has been the main focus for gene regulation in the past. However, a tremendous amount of evidence from recent studies also indicates that translational regulation plays a key role during development, cell cycle control, and mechanisms related to acute drug resistance. Gene expression analysis on actively translated mRNA transcripts provides a unique approach to studying posttranscriptional regulation. Previous studies have relied on a traditional sucrose gradient ultracentrifugation procedure to isolate polysome complexes and requires a large amount of cells (up to 500 million cells). As a result, this remains a major bottleneck for the investigation of posttranscriptional regulation with limited quantities of clinical samples. Therefore, there is an urgent need to develop a novel approach to isolate actively translated polysomes from a small number of cells (10 to 500 cells). The new approach will allow us to systematically study potential translational regulation with limited clinical samples. It has been shown that actively translated mRNAs are associated with multiple units of ribosomes and that the newly synthesized polypeptides are closely associated with molecular chaperones such as hsp73. These molecular chaperones assist in the proper folding of nascent polypeptides into higher ordered structures. These chaperones will provide the anchor to separate actively translated mRNAs associated with polysomes from free mRNAs. Affinity antibody capture beads will be developed to capture hsp73 chaperones associated with the polysome complexes so that all polysomes can be separated from monosomes and free mRNAs. The isolated actively translated mRNAs will be used for high-throughput gene expression analysis. The Specific Aims of the proposed project are to (1) develop antibody conjugated affinity capture magnetic beads and conditions to capture actively translated mRNAs associated with the polysome complex from a small number of cells, (2) validate the antibody affinity capture approach for polysome isolation by comparing with traditional polysome isolation protocols via quantitative RT-PCR

gene expression analysis, and (3) identify potential translationally regulated genes that are responsible for determining chemosensitivity during 5-fluorouracil (5-FU) treatment from human colon cancer samples.

Novel Method for Sample Processing

Trnovsky, Jan, One Cell Systems, Inc. 1 R43 CA120297-01 (SBIR)

Description: Development of improved procedures for sample preparation and storage of biological specimens is critical for diagnosis of human diseases, particularly cancer, for which positive cells are rare. Recent improvements in sample preparation and storage of cervical specimens led to significant increases in sensitivity and accuracy of cervical cancer screening. Although important, there have been few improvements in sample preparation, handling, and storage of other biological specimens (e.g., fine-needle aspirates or other biopsy materials). Problems associated with specimen handling include cell loss and cell clumping. Sample loss resulting from conventional biological specimen processing conditions such as fixation and permeabilization, used for intracellular immunophenotyping, is a well-documented phenomenon. If the sample is limited, or cells of interest are rare, cell loss is a significant problem hampering research, diagnosis, patient monitoring, and clinical studies. This SBIR aims to develop a simple procedure to reduce cell loss associated with sample processing and intracellular immunophenotyping, as well as cell clumping.

Integrated Microdevice To Capture and Detect Circulating Tumor Cells

Cote, Richard, Pathology and Urology Department, Keck School of Medicine, University of Southern California 1 R21 CA123027-01

Description: Metastasis is probably the most important event for determining outcome in cancer patients. The detection of occult metastases in the bone marrow, while known to be clinically important, has not become routine clinical practice. This is due to the technical difficulties and costs involved in the current methods for their collection and detection. Detection of circulating tumor cells (CTCs) in the blood is less sensitive than in bone marrow and suffers from the same technical barriers as the detection of tumor cells in the bone marrow, but offers the distinct advantage of being less invasive and better for patient compliance. Therefore, sensitive detection of earliest metastatic spread of tumor in a minimally invasive and user-friendly manner will have a great impact on the clinical management of cancer patients. The currently available methodologies for CTC capture and identification face significant barriers, including multiple procedural steps, substantial human intervention, extremely high cost, and, importantly, lack of reliability and standardization for the detection methods. We have demonstrated the potential for size-based tumor cell capture using a parylene-based micropore membrane. We propose to develop this into a microchip device for processing blood and eventually bone marrow and other fluids like pleural effusions or ascites. This microdevice, coupled with microfluidics, has the potential to revolutionize the approach to tumor cell capture and identification. Furthermore, we propose to develop methods for on-chip characterization of the captured cells. First, in the R21 Phase, we will develop and optimize the capture device using a model system to isolate and molecularly characterize cultured cancer cells admixed in blood, followed by a pilot study to examine blood from 45 actual cancer patients with metastatic disease for breast, prostate, or bladder cancer. In the R33 Phase, we will extend the application of the microdevice to assess about 310 patient samples from the same three malignancies, and we will also assess the molecular characteristics of the CTC using quantum dots to understand the biological features of these otherwise rare cells (such as existence of the putative stem cell subpopulation, which may be more malignant). At completion, studies in this project will develop a cost-effective on-chip system for capture, identification, and characterization of CTC, easily usable in the clinical setting.

Characterization of Methods for Preservation of Phosphoproteins in Fixed Tissues

Hsi, Eric D., Hematology Department of Clinical Pathology, Cleveland Clinic Foundation 1 R21 CA123006-01

Description: Protein phosphorylation is an important mechanism for regulating protein function and activity that depends on a competing system of kinases and phosphatases. It is a dynamic process that is altered in many disease states. For example, activated tyrosine kinases are central to the pathogenesis of chronic myelogenous leukemia (BCR-ABL1) and gastrointestinal stromal tumors(KIT). Detection of phosphoproteins (PPs) in fixed tissues by in situ immunohistologic methods may have diagnostic, prognostic, and therapeutic implications for cancer patients. Initial studies have shown that PPs are quite labile. Little is known regarding methods to preserve phosphorylation status in tissues. The purpose of this application is to develop optimal tissue-handling methods that will be suitable for detection of PPs in fixed tissues, keeping in mind practical limitations in the clinical setting. To this end, we intend to (1) develop a quantitative immunofluorescence (IF) method using quantum dots to quantitate PP status in fixed cell blocks; (2) characterize optimal fixation conditions (time, fixative, requirement of phosphatase inhibitors) in murine xenografts of human cell lines as a controlled model of available control material that is assayed both by quantitative Western blot and IF; and (3) show proof of principle in a murine model of BCR-ABL1 containing cell line xenograft treated with imatinib mesylate (IM) and bone marrow biopsies from patients suspected of chronic myeloproliferative disorder harboring the JAK2 V617F mutation. Phospho-STAT5 is known to be increased in both these systems. Decreased expression by phospho-STAT5 immunostaining in IM-treated xenografts and increased expression in JAC2 V617F+ bone marrow megakaryoctyes is expected in optimally handled tissues. This application has relevance in the diagnosis, prognosis, and therapy of malignancies and other diseases that have altered PP levels as part of their pathogenic pathways. It will define tissue-handling conditions that adequately preserve in vivo PP status for subsequent diagnostic and prognostic testing. Furthermore, control material with defined relative expression levels of many PPs will result from this application and allow laboratories to assess performance of their individual assays.

Microdevice for Direct DNA Purification

Landers, James P., Department of Chemistry, University of Virginia 1 R33 CA116115-01A1

Description: With an ever-increasing interest in the molecular typing of cells from histologic tissue sections, the ability to rapidly and efficiently extract DNA from the selected cells will be of paramount importance. The overall goal of this project is to develop a microchip-based sample preparation method for high-efficiency, low-cost extraction of DNA from tissue samples. This microdevice will easily accommodate blood or other cell sources. The microdevices will be created using state-of-the-art microfabrication techniques coupled with fluidically controlled on-chip cell lysis and solid-phase extraction chemistries. This project couples the industrial capabilities of HT Micro for facile fabrication of complex, high-surface-area microstructures with surface modification chemistries developed at the University of Virginia that enable efficient and high-capacity DNA extraction. The microdevices will be tested using a variety of samples varying in type and quality, and extraction efficiency will be determined using real-time PCR. As a demonstration of integration with current laser-capture microdissection instruments, the microdevice will be fabricated to directly accept the cap from the Arcturus PixCell IIe, which will contain the selected cells bound to an ethylene vinyl acetate polymer membrane on its bottom surface. The tissue samples will be collected and laser microdissected by our surgical pathology collaborator here at the University of Virginia, and samples of normal and malignant cells will be analyzed. The final device will offer the rapid-analysis, high-extraction efficiency, and high-throughput advantages of microdevices and, in addition, is expected to offer higher capacity and lower cost per device than current conventional or microchip techniques.

Validation and Quantification of FFPE Antigen Retrieval by Proteome Analysis

Balgley, Brian, Institutional Partner: Calibrant Biosystems, Inc., Academic Affiliation: University of Southern California 1 R41 CA122715-01 (STTR)

Description: Because of the long history of the use of formalin as the standard fixative for tissue processing in histopathology, there are a large number of archival formalin-fixed and paraffin-embedded (FFPE) tissue banks worldwide. These FFPE tissue collections, with the attached clinical and outcome information, present invaluable resources for conducting retrospective protein biomarker investigations. However, the high degree of covalently cross-linked proteins in FFPE tissues hinders efficient extraction of proteins from tissue sections and prevents subsequent bioanalytical efforts from opening the door to a veritable treasure trove of information sequestered in archival tissue banks. Thus, this project aims to address methodological optimization for achieving effective protein extraction from FFPE tissues together with technological development for performing comprehensive and comparative studies of protein expression profiles within FFPE tissue specimens. By combining Calibrant's ability to enable proteomic profiling from minute protein samples with the technology and expertise offered by Professor Clive R. Taylor (University of Southern California School of Medicine) in antigen retrieval (AR) immunohistochemistry (IHC) and tumor pathology, the proposed research represents a synergistic effort toward the evaluation and validation of a novel biomarker discovery paradigm on the basis of years of archived FFPE tissue collections. The Specific Aims for the R41 Phase and the respective scientific milestones are as follows: Specific Aim 1. Evaluation and optimization of protein recovery from FFPE tissues using AR approaches coupled with Gemini proteome platform (months 1-12). Scientific Milestone: Selection of an optimal protein recovery protocol to be employed in proteome investigations using a "test battery" AR approach and IHC staining for a panel of 10-20 proteins. Specific Aim 2. Validation and quantification of antigens retrieved from FFPE tissue specimens (months 13-24). Scientific Milestone: Demonstration of a sample consumption of 5 microgram total protein or less for an average of five peptides in each protein identification and the identification of at least 3,000 high-confidence proteins with greater than 80 percent reproducibility in identified proteins among triplicates of identical FFPE tissue using combined AR and Gemini technologies. Direct comparison of IHC results and proteomic display will be attempted for a panel of 10-20 proteins.

Improving Cancer Sample Preservation Through Analyzing Cell Stress Pathways

Baust, John G., Cell Preservation Services, Inc. 1 R43 CA118537-01A1 (SBIR)

Description: The intent of this project is to develop improved methods for the cryopreservation of human cancer cell lines and tumor biopsies. While there has been a current emphasis on the importance of improving biobanking of tissues (GEN, 2/1/05), there has been little research dedicated to improving the archival storage of cancer samples. Cell Preservation Services, Inc. (CPSI) develops hypothermic storage (HypoThermosol, "HTS") and cryopreservation (CryoStor) solutions marketed by CPSI's partner, BioLife Solutions. These solutions are used as the shipping/storage solutions in regenerative medicine applications such as cellular cardiomyoplasty. The fully defined, serum-free CryoStor platform is designed for cell and tissue storage in liquid nitrogen, requires reduced DMSO levels, and improves cryopreservation efficacy up to 50 percent compared to traditional protocols. Yet, even the CryoStor series can protect the viability of cancer cells only to a maximum of 70 percent. Thus, this "cryopreservation cap" must be attacked so as to achieve optimal preservation. CPSI is able to develop improved HTS and CryoStor solutions given its ability to investigate and modulate the cell death cascades that can be initiated due to extended storage. The purpose of this Phase I Project is to develop a new platform of solutions called CryoStor-CANCER, a series of solutions expressly designed for the improved cryopreservation of human cancer cells and tumor biopsies. CPSI will (1) use cDNA microarrays to determine whether the analysis of stress pathways activated by cancer cells undergoing cryopreservation can lead to improved preservation solutions for cancer cells and tumor biopsies; (2) determine whether the same analysis of stress pathways might lead to "rescue solutions" that can reverse the adverse effects of suboptimal cryopreservation; and (3) determine whether suboptimal preservation results in a long-term change to the expression of any of the 400+ genes that are most often studied in cancer biology. Phase II studies will test the prototype CryoStor solutions on a wider variety of cancer cell and tumor types and determine whether

cryopreservation and the proposed "rescue solution" preserve or rescue the oncological fingerprint of cancer cell lines and tumor tissues. This work will be important to agencies that are currently developing improved methods for biobanking cryopreserved cancer specimens. As a result, archival storage of cancer tissues will be improved, and poorly preserved specimens can be rescued.

FY 2007

Cryopreparation of Peripheral Blood Mononuclear Cells for Immunotherapy and Immune Assessment Studies

Yannelli, John, Microbiology, Immunology, and Molecular Genetics, University of Kentucky
1 R21 CA120693-01A2

Description: There are many immunotherapy trials being conducted throughout the world ranging from vaccines to T cell transfer and antibody studies. In these trials, aside from the obvious need to assess clinical responses, investigators must analyze changes in immune responsiveness that results from the immune intervention. Thus, there is a need to collect peripheral blood mononuclear cells (PBMCs) from patients at regular intervals both before and after therapy. These PBMCs are cryopreserved to save the PBMC phenotype and function until a later point, when the PBMCs can be thawed and assessed to determine whether the immunotherapy was effective. In addition, preparation of subsequent doses benefits from a source of readily obtainable PBMCs that will respond to in vitro manipulation similar to that on the day the product was received by the laboratory. There are few standardized techniques available for cell cryopreservation as it relates to immunotherapy. Most laboratories have their own protocols, which often provide inconsistencies in cell viability and function upon thawing. It is also difficult to evaluate the results of immunotherapy trials and make comparisons from laboratory to laboratory. The current proposal will focus on cryopreservation medium and compare static versus controlled rate freezing techniques. In four specific aims, we will examine the following: Specific Aim 1 will determine the optimum medium for cryopreservation. Human serum plus dimethylsulfoxide (DMSO) will be compared to Plasmalyte-A, a U.S. Food and Drug Administration (FDA)-approved, commercial, serum-free electrolyte or rehydration fluid. The comparison of Plasmalyte A to serum is important because, if it works, it will result in significant cost reduction, since the cost per liter is less than 1 percent of the cost of serum. In addition, this is an FDA-approved product that is consistent from batch to batch. Specific Aim 2 will evaluate different methods of thaw. Specific Aim 3 will compare static freeze techniques to controlled rate freezing. Specific Aim 4 will monitor Specific Aims 1 and 3 by comparing PBMC subset function at various time intervals, using assays of cellular immunity. The goal of the proposal will be to develop a strategy for PBMC cryopreservation that can be more universally applied. A more standardized approach will allow a more accurate assessment of immune responsiveness that can be compared between studies in different centers. In addition, other clinical studies utilizing PBMCs can also benefit from such a study (e.g., studies of HIV and transplantation). The four specific aims will be accomplished over 2 years.

Multiplexing Cancer Sample Preparation: Indirect Immunomagnetic Enrichment

Pamula, Vamsee, Advanced Liquid Logic 1 R43 CA132049-01 (SBIR)

Description: Biomarkers of clinical activity are critical for targeted anticancer therapy development and are becoming important for the care of individual patients. In a prototypical tyrosine kinase pathway, governed by the epidermal growth factor receptor (EGFR), functional activity of the pathway is assessed by the phosphorylation status of EGFR and downstream signaling intermediaries such as phospho-ERK and phospho-Akt. Interventions that block the function of EGFR (such as tyrosine kinase inhibitors or monoclonal antibodies) may lead to lack of phosphorylation of these downstream intermediaries. Traditionally, phosphorylated proteins have been analyzed by Western blots performed on tumor protein extracts from as many as 106 cells. A method is proposed that can utilize small samples to achieve a similar level of detection of phosphoproteins in the EGFR pathway. Tumor cells would be isolated from peripheral blood obtained before and after administration of an EGFR-targeted therapy, and the phosphorylation status of key EGFR pathway intermediates would be analyzed. The proposed approach to sample preparation is based on two molecular recognition events: (1) capturing analytes of interest (in this case, tumor cells, or their components after lysis) on nonmagnetic beads carrying receptors as well as "codes" and then (2) binding these beads by magnetic beads carrying "anticodes." The codes and anticodes can simply be two complementary DNA strands. Unlike single-step magnetic-bead capture, the proposed method allows simultaneous capture of multiple analytes by incubation with different bead types at the same time. They are then sorted by consecutive exposure to various types of

"decoding" beads. After the samples are processed to simultaneously capture multiple analytes of interest, the sample will be loaded onto an electrowetting (EW) biochip. The sample will be subdivided into droplets of similar size and run past droplets containing decoding magnetic beads. The ability of the EW chip to rapidly process multiple droplets enables the sorting procedure. For example, different aliquots of bead suspension can be reacted with the batches of magnetic beads in different sequences to avoid bias due to nonspecific binding. The ultimate advantage is sample concentration by at least 103-fold and removal of background material. The sample need not be subdivided, which increases the sensitivity and speed of multiplexed assays while allowing minimally invasive sample collection. Moreover, the final analysis—immunoassay, polymerase chain reaction (PCR), or reverse transcriptase PCR—can be performed on the same chip, taking advantage of the ultimate sensitivity and dynamic range of these liquid-phase assays. Advanced Liquid Logic, Inc., will team with collaborators at Duke University's Comprehensive Cancer Center to execute this project.

Collection, Stabilization, and Storage of Saliva Samples for Cancer Research

Wong, David, School of Dentistry, University of California, Los Angeles 1 R21 CA126733-01A1

Description: The ability to diagnose cancer at an early stage will greatly enhance the chances of treatment success and reduce mortality and morbidity. Currently there is no molecular test that can noninvasively diagnose or screen for oral cancer. We were able to identify a seven-gene signature that can predict the presence of an oral cancer with an accuracy of 82 percent. Since 2004, the characterization and diagnostic use of human salivary RNA have been actively pursued in our laboratory. Our proposal, "Collection, Stabilization and Storage of Saliva Samples for Cancer Research," responds to the RFA titled "Innovations in Cancer Sample Preparation" (RFA CA-07-037). We will develop optimal and standardized collection, RNA stabilization, storage, and processing protocols to perform gene expression analysis of salivary mRNA. We propose three specific aims to address the multifaceted nature of this RFA. Specific Aim 1 will establish optimal extraction methods for mRNA from saliva. In Specific Aim 2, we will determine the intra-individual fluctuation of mRNA levels, define adequate endogenous transcripts for normalization, and validate our oral cancer markers and additional new candidates in an independent cohort. In Specific Aim 3, we will determine the stability of saliva and expression patterns over periods of up to 6 months with different storage conditions and with stabilizing reagent. In all aims, we will apply a new method for the multiplex reverse transcription and pre-amplification with subsequent quantitative polymerase chain reaction (PCR) of a variety of mRNA transcripts endogenous to saliva. In addition, our setup allows the implementation of several spike sequences that will yield important insights into extraction and storage and are ideal controls for clinical application. We are confident that our research can be translated directly into a study for the large validation of oral cancer markers and spark the initiation of a multitude of projects and sample collections for the use of saliva as a diagnostic tool for oral and systemic diseases. Our studies will contribute to the advancement of human saliva as a clinically important body fluid for molecular diagnosis to improve cancer-directed health care.

Accelerating Cancer Research With Single-Cell Arrays

Weier, Heinz-Ulrich, University of California-Lawrence Berkeley National Laboratory 1 R21 CA132815-01

Description: This proposal addresses the sensitive detection of chromosomal changes such as small translocations, rearrangements, or genomic imbalances in apparently normal individuals, benign neoplasia, premalignant lesions, and cancer. Current techniques for full karyotype analysis of individual cells require metaphase cells, and cells in interphase or nonviable cells cannot be analyzed. Many cells that can be obtained from human tumors are not in metaphase. The objective of the proposed research is the development of technologies to support the cytogenetic analysis of small amounts of fresh, fixed, or archival tissues regardless of the cells' proliferative stage. A highly sensitive, fluorescence in situ hybridization (FISH)-based technology platform, termed single-cell arrays (SCAs), will allow the detection of small rearrangements in interphase and metaphase cells by combining the high-resolution DNA in situ analysis with sensitivity in the kilobase (kb) range. This will be achieved by immobilizing cell nuclei on glass slides and controlled stretching of chromatin in specially designed microchambers, followed by cytogenetic analysis using fluorescence in situ hybridization (FISH). The specific aims of this R21 feasibility study are as follows: (1) Demonstrate that interphase cell nuclei

can be immobilized in a defined pattern and reproducibly extended for subsequent cytogenetic analysis. We will demonstrate the feasibility of preparing SCAs comprising individual cell nuclei arranged in a defined pattern inside microscopic reaction chambers and elongated/stretched by a constant force. Importantly, the extent of chromatin stretching will be controlled by cell fixation and adjusting environmental parameters such as buffer, chamber temperature, and humidity, and the force applied to pull the chromatin. (2) Develop an optimized assay for the sensitive, high-resolution cytogenetic analysis of SCAs. We will develop a protocol for a FISH-based, multilocus, cytogenetic analysis of SCAs. The assay is expected to provide near-kilobase sensitivity for the detection of single-copy nucleic acids with a resolution in the order of 10-20 kb while minimizing the overall loss of DNA. The assay will be tested by analyzing SCAs prepared from different breast or thyroid cancer cell lines. SCAs will become powerful tools in basic and applied/clinical research, where chromosomal changes often affect a cell's phenotype and the fate of its progeny. In clinical practice, for example, such a sensitive assay may support cell classifications, thereby benefiting patients with de novo translocations or premalignant lesions as well as cancer patients. Furthermore, SCAs will allow the analysis of very small samples, regardless of their integrity or cell cycle stage. This will open new avenues for the analysis of small samples such as those obtained by fine-needle biopsies as well as the analysis of circulating or exfoliated tumor cells.

Tissue Lysates for Studies of Protein Phosphorylation

Knudsen, Beatrice, Department of Cancer Biology, Fred Hutchinson Cancer Research Center
1 R21 CA118592-01A2

Description: The long-term objective of this project is to measure phosphoproteins in human cancer tissues. As the first step toward this goal, the objective of this R21 grant is to identify the optimal conditions for preparation of tissue samples by testing multiple combinations of tissue fixation and protein extraction buffers. Xenograft tissues are frozen or fixed in a non-cross-linking fixative or in formalin. Proteins are extracted with four buffers and analyzed for protein phosphorylation by using a dot-blot assay. In Specific Aim 1, we measure global phosphorylation on tyrosine and threonine. We also use proQ Diamond to detect all phosphorylated proteins. In Specific Aim 2, we evaluate specific phosphorylation sites in proteins that are of clinical significance as drug targets or predictive biomarkers. We use statistical methods to identify sample preparation protocols that (1) are reproducible, (2) provide a high yield of extracted proteins, and (3) preserve protein phosphorylation during the extraction process. We rank the results and compare the top-ranked protocols to a reference sample preparation procedure. We anticipate that, by identifying sample preparation protocols for measurement of global and specific protein phosphorylation in cancer tissues, we will improve drug selection and response rates for many patients with solid tumors.

Validation and Quantification of FFPE Antigen Retrieval by Proteome Analysis

Balgley, Brian, Department of Proteomics, Calibrant Biosystems, Inc. 2 R42 CA122715-03 (STTR)

Description: Because of the long history of the use of formalin as the standard fixative for tissue processing in histopathology, there are a large number of archival formalin-fixed and paraffin-embedded (FFPE) tissue banks worldwide. These FFPE tissue collections, with attached clinical and outcome information, present invaluable resources for conducting retrospective protein biomarker investigations. In addition to sample amount constraints imposed by current proteome techniques, including two-dimensional polyacrylamide gel electrophoresis and multidimensional liquid chromatography system, the lack of optimized methodologies for retrieving proteins from FFPE tissues further restricts the ability to perform the molecular analysis of archival tissues. By collaborating with Drs. Shan-Rong Shi and Clive R. Taylor from the University of Southern California (USC) Keck School of Medicine during the R41 Phase I studies, the combination of antigen retrieval (AR) with Gemini proteomic technologies not only accomplished the rigorous evaluation of the quality and the reproducibility of proteins retrieved from FFPE tissues for the optimization of AR methodology, but also demonstrated significant opportunities in the pursuit of biomarker discovery using archived FFPE tissue collections. The proposed synergistic efforts between Calibrant and the USC team during the R42 Phase II project aim to generate proteotypic peptide libraries among model and tumor FFPE tissues. These

proteotypic peptide libraries represent the first step toward globally cataloging antigens retrievable from FFPE tissues and presenting the available epitope database for subsequent immunohistochemistry (IHC) antibody development. Besides providing guidelines for practitioners of IHC to select the optimized AR condition/antibody combination, the comparative proteomic and validation studies involving the use of proteotypic peptide libraries and associated antibodies will provide further enhancements in the reproducibility and the sensitivity of quantitative IHC measurements. The demand for quantitative IHC continues to escalate due to the widespread utilization of IHC in clinical diagnosis/prognosis and translational cancer research. Furthermore, the greatest expectations for targeted proteomics research using enriched and selected cells from high-quality specimens reside in the identification of diagnostic, prognostic, and predictive biological markers in the clinical setting and during preclinical testing and clinical trials, as well as the discovery and validation of new protein targets in the biopharmaceutical industry. The Critical Path Opportunity Report released by the U.S. Food and Drug Administration in March 2006 not only serves as the first specific blueprint for the Critical Path Initiative, an effort to streamline the drug approval process by applying new strategies and technologies, but also highlights biomarker development as one of the "most important areas for improving medical product development."

The Development of Cross-Platform Quantitative Standards for Diagnostic Immunohistochemical Staining

Camp, Robert, HistoRx, Inc. 1 R41 CA134335-01

Description: Patient-specific therapies targeted to the phenotype of individual tumors represent the future of cancer treatment. The prototypes for this type of therapy are trastuzumab (Herceptin) and tamoxifen, which are given only to patients expressing a particular level of HER2 and estrogen receptor (ER), respectively. It is likely that dozens of such therapies will be developed for a variety of tumors in the next decade. As with trastuzumab and tamoxifen, the diagnostic test for determining which patients will be given what therapies will most likely involve immunohistochemical (IHC) staining of fixed tumor samples, since the routine hematoxylin-eosin slide remains the standard for the anatomic diagnosis. Yet today's immunohistology laboratories have only the crudest of standards, usually a highly positive and a completely negative cell line or tissue control. The results from such stains are probably accurate to only at best one order of magnitude, even when newer automated quantitative stain readers are used. Furthermore, no attempt is made to assess the antigenic viability of patient samples. We believe that the future success of targeted therapy will be largely dependent upon the development of a robust method of standardization for IHC staining that can be used to normalize results across both laboratories and analysis platforms. Our goal is to develop a robust, adaptable IHC standard that can be processed alongside every patient tissue sample. This standard can then be used both to assess tissue antigenic viability and to permit the quantification of biomarkers at the molecules-per-cell level.

DEPARTMENT OF HEALTH & HUMAN SERVICES · USA

NATIONAL INSTITUTES OF HEALTH

NATIONAL® CANCER INSTITUTE