1st Principal Investigators Meeting Abstracts

1st Principal Investigators Meeting
Innovative Molecular Analysis Technologies Program
July 6-8, 2000
Westfields International Conference Center
Chantilly, Virginia

Abstracts

Ruedi Aebersold
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Quantitative Proteome Analysis: Methods and Applications

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A panel of powerful genomic technologies now permit the determination of complete genome sequences and the global measurement of mRNA gene expression profiles in cells. It is the premise of proteomics that i) the data obtained from genetic and genomic techniques by themselves are insufficient to describe physiological and pathological processes and that ii) the data obtained from the global analysis of gene expression at the protein level (proteome analysis) provide a better or complementary basis for the analysis of biological processes. In this presentation, three key issues related to proteome analysis will be discussed. First, we will examine to what extent gene expression at the mRNA and protein levels are correlated, i.e. to what extent protein expression levels can be predicted from genomic information. Second, we will assess current proteome analysis techniques and third, we will describe new technical approaches for quantitative proteome analysis which eliminate some limitations of the current standard techniques.

Leigh Anderson and Norman Anderson
Large Scale Proteomics Corporation
Proteomics Applied to the Search for Cancer Markers in Human Body Fluids

Leigh Anderson, Sandra Steiner, Rembert Pieper, Elling Eidbo, Tim Myers, Andy McGrath and Norman Anderson Large Scale Proteomics Corporation, Rockville, MD

Proteomics technology provides the opportunity to discover quantitative and qualitative protein markers of disease and drug effects. Using a multilevel approach involving fractionation, two-dimensional electrophoresis, and mass spectrometry, the abundance and structures of large numbers of proteins in biological samples can be investigated directly, and through comparison of appropriately structured sample sets, candidate markers can be detected. This approach has yielded a range of interesting insights and markers when applied to the analysis of drug effects in experimental animal models,

including the demonstration that drug mechanisms can be differentiated and classified using a large panel of protein markers. In the area of human disease, tissue protein markers have been found even in such difficult areas as psychiatric disease. However additional challenges arise in the systematic application of this technology to discover cancer markers in human body fluids. Primary among these is the fact that body fluids contain a series of high abundance proteins (e.g., the serum proteins) that are not expected to be useful markers, but which are present at abundances many orders of magnitude greater than most known potential markers. Since protein-resolving techniques such as 2-D electrophoresis typically have a useful dynamic range of only two to four orders of magnitude, it is necessary to use additional fractionation methods to deplete the most abundant molecules before 2-D analysis. We have succeeded in specifically removing the six most abundant human serum proteins, amounting to >75% of the total protein in serum, and have explored a series of other techniques for resolving the remaining proteins into fractions. These methods have allowed us to initiate a systematic search for protein markers present in serum and urine that are associated with specific cancers.

Plamen Atanassov
Superior MicroPowders

Novel Small Particle Phosphor Powders for Immunoassays

Mark Hampden-Smith and Plamen Atanassov Superior MicroPowders, Albuquerque, NM

Detection of histological markers for carcinomas has traditionally involved the staining of cells or cell markers with fluorescent dyes as indicators. However, autofluorescence is a common problem that ultimately limits the diagnostic utility of the results. This project attempts to overcome this limitation by the use of a reporter technology based on up-converting phosphor materials. This particle-based technology up-converts infrared to visible light and avoids the interference caused by background fluorescence, resulting in lower detection limits. Superior MicroPowders, in close collaboration with STC Technologies, is currently developing a spectrum of novel phosphor powders for use in phosphorescent immunoassays (PIAs). Superior MicroPowders' unique spray powder manufacturing technology allows for production of phosphors with control over morphology, particle size, and particle size distribution by spray routes. The background of this technology and the advantages it offers in producing a wide variety of particle supports/labels for immunoassays will be discussed. It is expected that the utility of up-converter phosphor powders will increase dramatically if they can be used as *in situ* probes for molecular detection by reducing their particle size below 400 nm and narrowing the spread of their size distribution. Prospective routes to achieve this goal will be discussed. Superior MicroPowders' materials engineering approach can be utilized in designing up-converting phosphors of different colors for simultaneous assay of several target analytes with enhanced sensitivity.

Cynthia Bamdad
Minerva Biotechnologies Corporation

The Electronic Detection of Interactions Between Ligands and Cell Surface Receptors

Cynthia Bamdad, Shoshana Bamdad, and Michael Frid Minerva Biotechnologies Corporation, Newton, MA

Minerva Biotechnologies has developed a revolutionary nanotechnology that enables the electronic detection of interactions between ligands and their cognate receptors on the surface of live, intact cells. The ligands can be natural ligands, antibodies, or drug candidates. The technique is so sensitive that it may enable single cell analysis. As the molecular mechanisms of cancer are unraveled, it appears that many of the pathways that lead to metastasis involve critical interactions with cell surface receptors. Our technology is of major importance because it is the first method that studies the cell in its native state and can detect interactions between surface receptors and natural ligands, as opposed to antibodies. Antibodies used as a probe can induce receptor clustering and initiate signaling cascades. Additionally, antibody-antigen interactions are not easily disrupted by drug candidates. Our method is totally electronic and is compatible with miniaturization and multiplexing. We will discuss the wide-ranging possibilities for application of this technology to the study of the molecular analysis of cancer and high-throughput drug screening to identify new therapeutics.

Robert Basedow and Douglas Kankel
Raytheon and MCDB, Yale University

Hyperspectral System for the Molecular Analysis of Cancer

Robert Basedow[1], Douglas Kankel[2], Michael Snyder[2], Peter Miller[1], and John Russo[1] [1]Raytheon, Danbury, CT, [2]MCDB, Yale University, New Haven, CT

Raytheon, in collaboration with Yale University, is developing an imaging spectrometer that will measure, in just seconds, the relative quantities of a mixture of up to 10 different fluorophore-tagged molecular species in each site of an array containing approximately 104 to 105 sites. It is primarily intended for use in the fields of cancer research and genomics, but it will also be capable of being used in a variety of applications, such as the evaluation of assays in high-throughput drug discovery experiments. This new spectrometer will be designed to be affordable to the average researcher versatile enough to measure all microarray formats now available or anticipated. The effort is primarily one of technology development and technology transfer. An imaging spectrometer design, of the type built by Raytheon for airborne surveillance and reconnaissance applications, will be adapted to the laboratory environment.

Challenges that have already been addressed in the aerospace world include maximizing throughput and sensitivity, minimizing optical distortion (especially "smile"),

minimizing scattered light, and ruggedizing the design. Data analysis techniques, which have been developed for unmixing the spectral components of a complex earth scene, will be applied to resolving the signatures in a mixture of fluorophores. These techniques are ideally suited to large data volumes in which both the target spectra and the background signal vary unpredictably. Given that the goal is to reliably measure more than 10 probes simultaneously, probe selection, and the optimal specific activity of selected probes, will be explored. To date the critical engineering issues involved in the design of an array scanner have been explored. Illumination and signal levels, bleaching thresholds and rates, spectral signature variability, and background levels have been measured on a breadboard using five probes selected, optimized, and fabricated for the purpose. The data have been used to verify both a radiometric model and the spectral unmixing algorithms. On the basis of this work a point design and system model for the prototype instrument have been produced.

Scott Bennett and Susan Castillo
SRA International
Combined Innovative Algorithms for Microarray Analysis

Scott Bennett, Susan Castillo and Eric Cahoon SRA International, Fairfax, VA

To better understand the vast quantity of microarray data currently being generated by numerous investigators, new computationally intensive analysis tools are needed. Pattern analysis of thousands of simultaneously expressed genes will provide insight into novel and progressive disease treatments. With the use of information technology tools, substantial opportunities exist for improving the ability to identify genetic anomalies. These tools will be critical for advancing the interpretation of experimental results through automation. We are developing a computer-based analytical tool called MultiCluster that will bring together multiple methods for analysis of microarray data. Each method has unique properties that produce conceptually different results. In the first phase of our work, we compare a number of clustering algorithms and their variants including hierarchical agglomerative techniques, K-means, and Kohonen maps. Cluster quality is measured through a combination of factors that determine how well known groupings are reflected in the output. Also measured are factors of efficiency and scalability. Using complex data sets such as those collected for the analysis of lymphoid gene expression, timed runs on expression datasets of varying sizes are reported. Currently, we are working towards giving scientists a unified view of the best overall clusters produced by several algorithms. Such a hybrid approach will allow scientists to quickly identify high-value consensus clusters for further investigation utilizing a combination of the discovery algorithms.

Aaron Bensimon
Pasteur Institute, DNA Biophysics Laboratory
Detection of Genomic Alteration by Molecular Combing

Aaron Bensimon[1], John Herrick[1], Philippe Passero[2], and Etienne Schwob[2] [1]Pasteur Institut, DNA Biophysics Laboratory, [2]Institute of Molecular Genetics, DNA Replication and Genome Stability Laboratory, Paris, France

Our research concerns the mechanisms underlying the control of DNA replication and genome stability with particular emphasis on the mechanisms and consequences of oncogene amplification. We have developed a new technology for the genomic study of DNA replication and genetic alterations. This technology involves a method called molecular combing, which can straighten and align molecules of genomic DNA on a solid surface. The technology also includes a battery of novel statistical methods developed for analyzing the large amounts of data obtained from FISH analyses made on individual DNA molecules.

Molecular combing relies on the action of a receding air/water interface, or meniscus, to uniformly straighten and align DNA molecules on a solid surface. The advantages of this approach reside in the reproducibility of the results, their precision (1 to 4 kb resolution) and the relative ease of analysis afforded by the ability to visualize the molecules directly. Beyond its obvious applications to genomic studies and genetic diseases, it creates new experimental possibilities for research into cancer. Indeed, as a tool, molecular combing is a versatile approach to a wide range of subjects and questions of fundamental interest. This is especially true for the multifaceted domain of DNA replication in eukaryotes.

We have demonstrated that this is an attractive approach to examine phenomena underlying the mechanism of carcinogenesis: i) The spatial pattern organization of the overrepresented MET regions was visualized and characterized as a cell signature in renal carcinoma. Moreover, the evolution of the overrepresented regions could be followed together with the progression of the tumour, showing a correlation between genomic instability and tumour progression. ii) Molecular combing, in combination with other higher resolution techniques, allows for the identification and mapping of origins of replication on a genome-wide basis. Consequently, the replication programs of higher eukaryotes can be reliably elucidated by determining the distribution and activities of replication origins over wide regions of the genome. Hence, our ultimate interest is to establish integrated replication/transcription maps of different regions of the genome at different stages of carcinogenesis.

The longer-term goal of our research is to elaborate a novel approach based on the analysis of individual DNA molecules to define how replication programs differ from one tissue type to another depending on the respective cells transcription profiles. The objective is to investigate those factors that are implicated in the aberrant replication of the genome in transformed cells and to elucidate the mechanisms underlying the replication programs in both normal and cancerous cells.

Steven A. Bogen
CytoLogix Corporation

Automation of *in situ* Hybridization to Tissue Sections and Arrays

Steven A. Bogen[1], Sandhya Raja[1], Herbert H. Loeffler[2], and Scott Leon[1] [1]CytoLogix Corporation, Cambridge, MA and [2]Loeffler-MacConkey Design, Winchester, MA

Modern methods for the genetic analysis of cancer include hybridization assays to tissue sections, cells, and arrays. These techniques are generally performed on an optically clear flat surface, such as a microscope slide. They require that a drop of reagent is spread over a planar surface and heated while preventing evaporation. Traditionally, this has often been accomplished by sealing a coverslip over the sample. However, that process is not easily adaptable to automation. Under an NCI SBIR grant, we have developed a microfluidics technology for a capillary-thick planar chamber. The chamber completely encloses and seals the liquid reagent in place, over the sample. Previously, the main technical hurdle with such miniature chambers was in exchanging the reagent, as is necessary in multi-step procedures. Specifically, it has been difficult to consistently fill capillary planar chambers with reagent without occasionally entrapping air bubbles over the sample. We have now overcome that problem by developing a method of forcing fluid reagent into a planar capillary-thick microchamber by laminar flow. Reagent enters the chamber in response to a hydrostatic pressure pulse. Small differences in fluid drag (such as by a tissue section) do not disrupt laminar flow through the chamber and therefore do not lead to bubble entrapment. The pressure pulse can take the form of either negative (vacuum) or positive pressure. We are currently testing this microfluidics technology for *in situ* hybridization and immunohistochemistry. Future efforts will focus on marrying this fluidics technology with a random access, walk-away automation capability, including independent sample temperature control, such as found on the CytoLogix ARTISAN instrument.

Graham J.R. Brock
Division of Molecular Genetics, IBLS, University of Glasgow

Identification of m5CpG Alterations in Breast Carcinomas

Graham J.R. Brock Division of Molecular Genetics, IBLS, University of Glasgow, Glasgow, Scotland, UK

The proposed research will identify methylation changes that occur during the development of human breast cancer. Such changes have been reported in GC-rich CpG islands during the development of a significant subset of breast cancers. Aberrant methylation patterns have also been reported in other GC-rich regions, for example in satellite or repetitive DNA. While it is unclear if such alterations are a primary cause or occur as a secondary effect, changes to the methylation patterns, once established, are thought to be faithfully reproduced in progeny cells. In order to identify these alterations a Methyl Binding Domain (MBD) column will first be used to isolate GC-rich methylated sequences from matched pairs (of both normal and neoplastic tissue) at different stages of development. Subtractive hybridization will then isolate those GC-rich sequences that display divergent methylation patterns between each matched pair. These sequences

will then be examined using a variety of molecular techniques. We hope to identify both hyper and hypomethylation changes during tumor progression and the earliest modification(s). Identification of the scope and scale of these modifications will enable earlier diagnosis and thus increase the efficacy of subsequent treatment.

Tauseef R. Butt
LifeSensors Inc.

Molecular Profiling of Disease by Functional Microarrays Composed of Human Genes in Yeast

Tauseef R. Butt, Michael Schwatrz, and Hiep L. Tran Research and Development, LifeSensors Inc, Malvern, PA

Molecular profiling of individuals holds immense promise in identifying individuals with high risk for progression of a specific type of cancer and quantifying response to therapy. Ligand-dependent functions of many human nuclear receptors have been established in yeast. Many of the receptors and their ligands are important therapeutic and diagnostic markers. The distinguishing feature of our technology is that yeast was engineered with several human nuclear receptors where the receptor function is driven by the ligand. Receptor-regulating activities from human sera of control and cancer patients can be monitored using the panel of functional microarrays called LiveSensors™. We have used the human estrogen receptor as a model system to develop an ultra-sensitive estrogen sensor. It has been shown that the order of potency for 17-ß-estradiol and phytoestrogens transactivation rank order of potency was remarkably similar in yeast and human HepG2 cells. Yeast cells demonstrated an order of magnitude higher efficacy for a variety of compounds as compared with human cells. Application of this technology to monitor predisposition to disease and therametrics will be discussed.

Jeffrey J. Chalmers
Department of Chemical Engineering, Ohio State University

Immunomagnetic Cell Separation for Rare Cancer Cell Detection in Blood: Current Applications and Future Potential

Jeffrey J. Chalmers[1], Masa Nakamura[1], Keith Decker[1], Julia Chosy[1], Kristie Melnik[1], Kara McCloskey[1], Lee Moore[2], Maciej Zborowski[2] [1]Department of Chemical Engineering, Ohio State University, [2]Department of Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, OH

Magnetically based cell separation technologies have become commonly used techniques to enrich and/or separate cells of interest from a heterogenous cell population. Recently, significant interest has been generated in the potential to separate rare cancer cells from blood. The analysis of this separation approach can be broken down into three aspects: 1) the specificity and selectivity of the immunomagnetic labels

for the target cell of interest; 2) the ability of the paramagnetic label to impart on the target cell a magnetophoretic mobility sufficient to allow an effective separation; and 3) the effectiveness of the actual separation system to remove the immunomagnetically labeled target cell from a heterogeneous mixture of cells. This presentation will attempt to summarize the current state of knowledge in each of these three aspects. In addition to reviewing the commercial systems currently available, a system currently under development which has the potential to significantly increase the throughput of cells in a short period of time will be presented.

Mark R. Chance
Center for Synchrotron Biosciences, Albert Einstein College of Medicine
Probing the Dynamics of Macromolecular Interactions by Protein Footprinting

Mark R. Chance, Simin Maleknia, and Sharon Goldsmith Center for Synchrotron Biosciences, Albert Einstein College of Medicine, Bronx, NY

Deriving detailed structure and dynamics information for macromolecules and their complexes is a challenging and important step towards providing a molecular understanding of normal and malignant cells. Although existing biophysical methods such as fluorescence, single molecule techniques, crystallography, and NMR have advanced our understanding greatly, it has been difficult to achieve high structural resolution, fast-time resolution, and the ability to monitor large assemblies while utilizing small amounts of precious samples. Time-resolved synchrotron x-ray footprinting is a relatively new technique developed to study the dynamics of nucleic acids. The method probes the solvent accessible surface of macromolecules and their complexes using hydroxyl radicals. The technique is coupled to stopped-flow initiation of reactions, and dynamics on timescales as fast as 5 milliseconds have been probed for nucleic acids. We have developed a quantitative hydroxyl radical footprinting technique using synchrotron radiation to probe the structure of proteins. Our successful examination of protein folding and protein-ligand complexes provide initial studies to bridge to more complex problems, namely detailed examination of interactions of large macromolecular complexes. Specifically, the focus will be methodologies to examine the detailed pair-wise interactions of large binary complexes from the individual perspective of each member of the pair. The model systems used to develop the technology include: 1) examining the orientation and binding of cofilin and actin, 2) examining the time-resolved dynamics of actin filament disassembly catalyzed by gelsolin, and 3) probing the time-resolved dynamics of reverse transcriptase. These model systems will drive the technology to provide general methods relevant to studying a wide range of problems in cancer biology. Time-resolved protein footprinting, when perfected, will be applicable to studying macromolecular interactions critical to replication, transcription, signal transduction, translation, processing, and secretion.

Mark Chee
Illumina, Inc.

Randomly Self-Assembled Bead-Based Arrays for Highly Parallel Analysis of Nucleic Acids

Mark Chee[1], Jian-Bing Fan[1], Shawn Baker[1], Tim McDaniel[1], Jonathan BenDor[1], Todd Dickinson[1], Chanfeng Zhao[1], Gan Wang[1], Joanne Yeakley[2], Xiang-Dong Fu[2], and Kevin Gunderson[1] [1]Illumina, Inc., San Diego, CA and [2]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA

Self-assembled arrays of bead-based sensors have been developed. Advantages include intrinsic flexibility, high information density, and access to a wide range of chemistries. Individual beads are 3 to 5 microns in diameter, and typically ~50,000 beads are randomly arrayed in a 1.2 x 1.2 mm area. A multicolor labeling scheme is used to decode the beads. The development of these arrays for RNA profiling and other genomics assays will be discussed.

S.L. Dabora
Division of Hematology, Brigham and Women's Hospital

Comprehensive Mutation Analysis Using DHPLC and Long Range PCR for Tuberous Sclerosis Complex, a Tumor Suppressor Gene Disorder

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the development of benign tumors (hamartomas) in multiple organs, often causing serious neurologic impairment. Two disease genes, TSC1 and TSC2, have been identified, but the development of comprehensive mutation analysis has been slowed by the large sizes of the two genes and a diverse mutation spectrum. We have developed a comprehensive strategy for identifying mutations that cause TSC. This strategy involves two components. First, exon-by-exon analysis of TSC2 and TSC1 using denaturing high performance liquid chromatography (DHPLC) scans for small mutations. Second, long-range and quantitative PCR assays identify large deletions in TSC2.

These methods are being used to identify mutations in TSC1 and TSC2 in a cohort of 228 TSC patients (approximately 87% sporadic cases and 13% familial cases), the majority of which are referred from Pediatric Neurology practices. The analysis is complete in 167 patients, but still in progress in the remaining 61 patients. So far, 143 mutations have been identified. This includes 122 TSC2 mutations and 21 TSC1 mutations. In TSC1, there are 7 nonsense mutations, 13 deletions, and 1 insertion. In TSC2, there are 28 nonsense mutations, 27 deletions, 15 insertions, 25 splice site mutations, and 27 missense mutations. Four of the TSC2 deletions were large (1.4 kb-

3.9 kb) and were identified using long-range PCR. Details of DHPLC analysis, long-range PCR, and quantitative PCR will be presented. Genotype/phenotype analysis in this cohort are underway.

Norman J. Dovichi
Department of Chemistry, University of Alberta, Canada
The Single Cell Proteome Project

Norman J. Dovichi, Zheru Zhang, Sergey Krylov, Edgar Arriaga, Shen Hu, and David Michels Department of Chemistry, University of Alberta, Alberta, Canada

A simple proteome map was obtained from single HT29 human colon adenocarcinoma cells. The cell of interest was introduced into a fused-silica capillary and lysed, and the protein content was fluorescently labeled. The labeled proteins were separated by capillary zone electrophoresis in a sub-micellar buffer and detected by laser-induced fluorescence in a post-column sheath-flow cuvette. Several dozen components were resolved. One component was identified as a 100 kD protein by co-injecting the purified protein obtained from an SDS-PAGE gel. Protein expression varied significantly between cells. This expression difference was correlated with cell cycle.

Bevin Engelward
Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology
Fluorescence Detection of Genetic Instability in Mice

Bevin Engelward[1] and Peter So[2] [1]Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, [1]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Should the remaining wild-type copy of a tumor suppressor gene become mutated in a heterozygous cell (referred to as loss of heterozygosity or LOH), the cell can become prone to tumorigenesis. Mitotic recombination has been estimated to be the underlying cause of LOH 25-50% of the time. In addition, mitotic recombination between misaligned sequences of the same chromosome can cause large deletions. Given that mitotic recombination causes large deletions and also drives cancer-promoting LOH events, it is critical that we learn more about the factors that modulate susceptibility to mitotic recombination. Our goal is to combine genetic engineering with mechanico-optical engineering to develop the technology to detect genetic instability in mammals. A transgenic mouse will be engineered to carry a fluorescent marker for the identification of cells that have undergone a mitotic recombination event. A high-throughput two-photon microscope system will make it possible to quantify recombinant cells *in situ* in a variety of cells, to characterize the cell types most prone to mitotic recombination, and to discern the contribution of recombination events that occur in stem cells. Yet another important application will be in studying the effects of chemotherapeutics on mitotic recombination and in determining how specific genetic

traits effect cellular susceptibility to chemotherapy-induced mitotic recombination. We hope that this new tool will be of fundamental importance in revealing genetic and environmental processes that drive cancer-promoting mitotic recombination events in mammals.

Terry Gaasterland
The Rockefeller University

Structural Genomics Target Selection for Cancer-Related Proteins Across Complete Genomes

Terry Gaasterland, Gulriz Aytekin-Kurban, Ursula Pieper, Roberto Sanchez, and Andrej Sali The Rockefeller University, New York, NY

Our goals are to identify and annotate cancer-related protein sequences from completely sequenced genomes; to organize them into sequence and structure families; to prioritize target proteins for the structural genomics of cancer; and to maximize structural information about cancer-related proteins. We identify "cancer-related" protein sequences in a four-step search of the public sequence databases using functional keyword searches, identification of genomic homologs, and identification of putative co-transcribed proteins in prokaryotes.

Next, we generate queryable and up-to-date annotations of cancer-related proteins through sensitive comparisons to all known protein sequences and structures. The annotations include assigned functional category, cellular role, and specific function where known. In addition, the genomic proteins are augmented by data about their location within the genome and the confidence level of assigned putative function. We calculate and evaluate comparative protein structure models for as many cancer-related proteins as possible. This structure data is augmented by information about folds extracted from the CATH, SCOP, and DALI databases. The annotation modeling and analysis tools build on the MAGPIE system for automated genome annotation, which is in active use for multiple genome sequencing projects, and on the MODELLER pipeline for large-scale comparative modeling. Annotation rules are defined through logical rules and relational facts implemented in Prolog, including rules to capture computed alignment data, domain definitions, and user preferences about properties of target domains.

The ability to refer at the same time to the sequence, structure, and function of cancer-related proteins organized in sequence and structure families will allow cancer researchers to address questions that are currently not easily answered. The current system (as of July, 2000) focuses on the comparative analysis and structure modeling of cancer-related proteins predicted from the Drosophila melanogaster complete genome.

John Gerdes
Xtrana, Incorporated

Measurement of Relative Levels of Estrogen Receptor (ER) Gene Expression in Breast Cancer Cells Utilizing Competitive Genome Quantitative PCR (CG-QPCR)

John Gerdes, Jeffrey Marmaro, and Craig Sampson Xtrana, Incorporated, Denver, CO

Xtrana is developing a sensitive and precise method for the relative measurement of gene expression following solid-phase capture of nucleic acids onto our proprietary Xtra BindTM solid phase matrix. Capture occurs in a PCR tube that is used directly for CG-QPCRTM amplification and detection. CG-QPCRTM utilizes specific primer and probe sets to detect the genome of the target gene of interest as the internal competitor for quantitative measurement of that gene's expression.

DNA and RNA are simultaneously yet independently measured using homogeneous fluorogenic detection probes (TaqManTM). This strategy provides a means of normalizing measurement fluctuations due to cell number, pseudogene sequences, specimen fixation, nucleic acid extraction, RNA degradation, and PCR amplification efficiency. It also permits RNA measurements using total nucleic acid extracts, eliminating the necessity for a RT minus control. ER gene processing is extremely complex with a number of alternative mRNA transcript isoforms. For ER CG-QPCRTM a common reverse primer was designed in a region common to both genomic DNA and all RNA transcripts. A forward primer was placed far upstream, in a region not contained in any transcript, to serve as the DNA-only primer. A nested forward primer was placed in a region near the reverse primer at a site contained in all known transcripts. The resulting amplification products correspond to DNA-only amplification and DNA plus RNA amplification. Changes in gene transcription rates are reflected in changes in the RNA+DNA/DNA-only amplification product ratios. Preliminary results indicate that ER CG-QPCRTM is capable of measuring gene expression with as few as 100 cells following total nucleic acid extraction and solid phase Xtra BindTM capture.

Roger W. Giese
Bouve College and Barnett Institute, Northeastern University
New Methodology for the Analysis of DNA Adducts

Roger W. Giese, Poguang Wang, Gang Shao, Xiaohua Qian, Aijian Liu, Yasser Ismail, and Jie Yao Bouve College and Barnett Institute, Northeastern University, Boston, MA

Nearly all of the substances that are classified as human carcinogens cause direct or indirect damage to DNA (DNA adducts). We are working on the development of a new methodology for the analysis of DNA adducts in human samples. Current methodologies for this purpose are limited, especially in the identification of unknown DNA adducts and in the definitive detection of multiple adducts simultaneously. Both of these features are important to assess multiple chemical exposure. In order to establish high sensitivity uniformly for a diversity of DNA adducts, we are relying on chemical tagging with an electrophore, dye, or cation label. In the case of dye labeling, final detection depends on capillary electrophoresis with laser-induced fluorescence

detection (CE-LIF). All three labels can facilitate detection by mass spectrometry (MS). Sample preparation, including the development of new reagents and techniques for efficiently converting DNA adducts into sensitive derivatives, is the main focus of our work. Phosphate-specific labeling of DNA adducts with an IMI dye followed by CE-LIF, labeling of sugar oxidation products with the new electrophoric reagent AMACE1 followed by GC-EC-MS, and KO2 transformation-electrophore derivatization of PAH DNA adducts followed by GC-EC-MS are examples of methodologies under study in our laboratory. Improved measurements of DNA adducts in human samples may lead to advances in cancer prevention.

Jeffrey Griffith
University of New Mexico School of Medicine

Telomere Length as an Independent Prognostic Parameter in Advanced Prostate Cancer

Jeffrey Griffith[1], Colleen Fordyce[1], Christine Stidley[2], Anthony Smith[3], and Nancy Joste[4]
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We have previously described a robust titration assay that can quantify telomere DNA content in as little as 15 ng of total DNA. Briefly, DNA is extracted from fresh, frozen, or paraffin-embedded tissues, fixed to nylon membranes, and hybridized with 32P-labeled centromere- and telomere-specific probes. Telomere DNA content is quantitated from the ratio of telomere and centromere probe radioactivities hybridized to each specimen to those hybridized to a reference, human placenta DNA included on each blot. We have used this assay to test the hypothesis that telomere shortening is correlated with poor clinical outcome in breast and prostate cancer. Our previously published studies have demonstrated that reduced telomere DNA content in invasive human breast carcinomas is associated with aneuploidy and metastasis ($p<0.002$, $p<0.05$ respectively) and in prostate adenocarcinoma with disease recurrence and death ($p<0.0001$, $p<0.0001$, respectively). However, this promising technology will only be useful in guiding clinical decisions if it can be applied to the limited samples available from needle-core biopsies. The purpose of the initial phase of this project is to demonstrate the feasibility of using this assay for measuring telomere DNA content in prostate needle-core biopsy specimens. In this context, we can now detect telomere DNA in the equivalent of 300 tumor cells (< 1 ng total DNA) by modifying the assay to utilize very sensitive chemiluminescence-based probe detection systems. In the second phase of this project, we will use the modified assay to perform a retrospective analysis of the relationship between patient survival and telomere DNA content in prostate needle-core biopsy specimens.

Bassem R. Haddad
Georgetown University Medical Center

Molecular Cytogenetic Screening of Mammary Epithelial Cells in Nipple

Bassem R. Haddad Georgetown University Medical Center, Washington, DC

Because optimal methods have not been established for screening and early detection of breast cancer in premenopausal women, there is an urgent need for a variety of new approaches that can augment mammographic screening. We propose to study a new noninvasive approach to early detection that is based on cytogenetic analyses using comparative genomic hybridization (CGH) of mammary epithelial cells shed into nipple aspirate fluid (NAF). This approach is based on the premise that women with early stages of neoplastic progression will have cytogenetic abnormalities (DNA gains and losses) that can be detected in the cells shed into NAF.

We propose to validate this technique by (1) demonstrating that we can successfully grow expand NAF-derived cells in culture to produce sufficient DNA for CGH following universal PCR amplification, (2) detecting differences in cytogenetic profiles of these cells between women with normal breasts and those with stage I breast cancer (35 women in each group), and (3) showing that cytogenetic abnormalities detected by CGH in NAF-derived cells from stage I breast cancer patients are consistent with those detected by CGH in tumor tissue from the same patients. If this approach is effective, it can be performed as a safe and noninvasive adjunct to mammography for breast cancer surveillance, particularly in young premenopausal women (<40) with an inherited predisposition to breast cancer who need early and continuous surveillance.

Amy-Joan L. Ham
Departrment of Pathology, University of North Carolina

Ultrasensitive Methods for Human DNA Adduct Quantitation

Amy-Joan L. Ham[1], Asoka Ranasinghe[2], Hasan Koc[2], Quoi Zhan[3], J. Ronald Hass[3] and James A. Swenberg[1,2] [1]Departrment of Pathology and [2]Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC and [3]Triangle Laboratories, Durham, NC

DNA adducts are believed to be a major source of mutations involved in carcinogenesis. The availability of innovative technologies for investigating the presence of these adducts will greatly aid basic research, epidemiological and chemoprevention studies, risk assessment, and occupational health. We propose to develop technologies for the routine analysis of DNA adducts that arise from medical, environmental, and occupational exposures, as well as from endogenous processes.

The primary focus of our proposed research is the development of ultrasensitive techniques for monitoring DNA adduct formation using liquid chromatography/electrospray tandem mass spectroscopy (LC-MS/MS). Specifically, we will develop LC-MS/MS methods for 3N2-ethenodeoxyguanosine, 1N2-ethenodeoxyguanosine, 1N6-ethenodeoxyadenosine, 3N4-ethenodeoxycytosine, 3-

methyladenine, 7-hydroxyethylguanine, 7-methylguanine, and possibly a number of propano, exocyclic guanine adducts derived from acrolein and crotonaldehyde. These adducts are formed in the DNA of animals and humans exposed to vinyl chloride or simple alkylating agents, and most can be demonstrated in DNA of unexposed animals and humans. Following the development of LC-MS/MS methods they will be compared with currently developed GC-MS methods for several of these adducts. Preliminary results with standards show that LC-MS/MS analysis of 3N2-ethenodeoxyguanosine (OGua) gave a signal-to-noise ratio (S/N) of 30 when 1 fmol is injected. This technology, coupled with immunoaffinity chromatography that results in 80-90 % recovery of OGua from DNA samples, suggests that this is a very feasible technology for the analysis of this adduct. In addition, LC-MS/MS analysis of 7-hydroxyethylguanine (7HEG) standards gave a S/N of 38 with the injection of 7 fmol.

Tim Hui-Ming Huang
Departments of Pathology and Anatomical Sciences, Ellis Fischel Cancer Center, University of Missouri School of Medicine

High-Throughput Methylation Analysis Using CpG Island Microarray

Tim Hui-Ming Huang, Martin R. Perry, and Pearlly S. Yan Departments of Pathology and Anatomical Sciences, Ellis Fischel Cancer Center, University of Missouri School of Medicine, Columbia, MO

Aberrant DNA methylation has been observed in GC-rich CpG island regions, which are frequently located in the promoter and first exon of genes. This type of epigenetic mutation is associated with the silencing of tumor suppressor genes and plays an important role in promoting tumor development. Until recently, most methylation assays have been limited to analyzing a few CpG islands of known genes at a time, and they therefore suffer limited throughput for a genome-wide study and for clinical application. Therefore, the development of more efficient technology designed to detect CpG island hypermethylation has long been needed to dissect complex methylation changes in cancer.

With this in mind, we recently developed Differential Methylation Hybridization (DMH), a microarray-based approach that simultaneously detects DNA methylation in thousands of CpG islands. The first part of DMH involves the generation of multiple CpG island tags as templates arrayed onto solid supports (glass slides or nylon membranes). The second part involves the preparation of amplicons that represent a pool of methylated DNA from the tumor and reference (control) genomes. Amplicons are used as probes in array-hybridization. Positive signals identified by the tumor amplicon, but not by the reference amplicon, indicate the presence of hypermethylated CpG island loci in cancer cells. DMH has been applied to screen 28 paired primary breast tumor and normal samples. Close to 9% of 1,104 CpG island tags analyzed exhibit hypermethylation in the majority of breast tumors relative to their normal controls, whereas others had little or no detectable changes. Methylation profile analysis indicates that poorly differentiated tumors appears to exhibit more hypermethylation than their moderately- or

well-differentiated counterparts (P=0.041). This early finding lays the groundwork for a population-based DMH study and demonstrates the need to develop a database for examining large-scale methylation data and for associating specific epigenetic signatures with clinical parameters in cancer.

Jonathan W. Jarvik and Robert F. Murphy
Department of Biological Sciences, Carnegie Mellon University
High-Throughput Functional Proteomics Using CD Tagging and Automated Image Analysis

Jonathan W. Jarvik and Robert F. Murphy Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

CD-tagging is a molecular-genetic technology that adds unique molecular tags to gene, transcript and protein, thereby allowing genes and proteins to be rapidly identified and visualized in their natural cellular and/or organismal environments. The technology also enables direct observation of changes in protein abundance, location, and structure as the cell traverses the cell cycle or as it responds to changes in the external or internal environment. In the present project, custom-designed retroviral vectors are used to deliver special CD-cassettes to random sites in the genomes of live cells. Insertion of the vector in the appropriate orientation in an intron of an expressed gene results in the addition of unique guest exons to the mRNA and the addition of unique guest peptides to the encoded protein.

To facilitate detection of tagged proteins, we use two kinds of guest peptides-epitope tags recognized by existing high titer antibodies and GFP tags. Tagged proteins are readily observed in live cells and are also readily affinity-purified for functional and biochemical analysis, including mass spectrometric identification. Analysis at the RNA and DNA levels is also straightforward, since the unique tag sequences in mRNA and DNA may be used to recover and sequence the nucleotides contributed by the native gene. As an important adjunct to CD-tagging, we have developed automated methods for analyzing subcellular location via fluorescence microscopy. Our current neural network-based system can correctly identify the subcellular patterns of all major organelles in cultured cells and can classify clones resulting from random CD-tagging. Using this automated system, we are generating a set of knowledge bases for normal and malignant cells that link protein localization data to genetic, biochemical and physical data for all members of the proteome that be detected in tagged form.

Daniel G. Jay
Department of Physiology, Tufts University School of Medicine
A High-Throughput Screen for Cancer Cell Invasiveness Using Chromophore-Assisted Laser Inactivation

Daniel G. Jay, Takashi Sakurai, Jean Stewart, Brenda Eustace, and Patrick Finn

Department of Physiology, Tufts University School of Medicine, Boston, MA

A major challenge in drug discovery is to identify proteins that have essential roles in cancer metastasis. There is a current lack of technology that addresses protein function directly, as most functional inactivation approaches target genes or mRNAs. We have developed and tested a high throughput assay for tumor cell invasiveness that employs Chromophore-Assisted Laser Inactivation (CALI) to inactivate proteins of interest in cancer cells to address their roles in invasiveness. CALI targets laser light to cause transient and localized loss of any protein bound by a specific probe (e.g. an antibody) that has been labeled with a photosensitizing chromophore.

Major advantages of this approach include: 1) the ability to target proteins that have essential roles early in development; 2) the ability to address protein function in human cells of disease relevance; and 3) the capacity and relative ease of mutiplex approaches (compared to gene knockout). To show that CALI enhances the probability of inactivation we have used single chain Fv (ScFvs) molecules (from Xerion Pharmaceuticals) that bind to the enzyme a-galactosidase. In the 7 cases tested, none of them inactivated a-galactosidase alone, but all of them showed significant inactivation upon irradiation with laser light. We have markedly improved a transwell invasiveness assay to allow for high-throughput screening and shown that it can be coupled to CALI. We have tested the efficacy of this screen using antibodies against a 1 integrin (a validated target for invasiveness). A model assay using 100 antibodies directed randomly against antigens expressed by HT-1080 cells is underway. Progress of this screen will be reported. We believe that this screen will be useful in identifying novel protein targets involved in invasiveness and that similar screens may be adapted for other processes important for cancer.

Tanya Kanigan and Sandra M. Gaston
Massachusetts Institute of Technology and Beth Israel Deaconess Medical Center

Androgen Receptor Bio-Chips: Novel Micro-Bioassays for Androgens and Androgen Mimics in the Sera and Tissues of Men with Prostate Cancer

Tanya Kanigan[1], Sandra M. Gaston[2], and Ian W. Hunter[1] [1]Massachusetts Institute of Technology, [2]Beth Israel Deaconess Medical Center, Cambridge, MA

The major objective of this project is the design and fabrication of a battery of micro-scale yeast-based bioassays in a "biochip" format that can be used to monitor androgen receptor (AR) ligands in sera and tissue extracts from patients with prostate cancer. Initially, prostate cancer is almost always an androgen-dependent disease, and androgen ablation has been a mainstay of treatment for men with metastatic prostate cancer since the 1940fs. However, major recent advances in the clinical management of prostate cancer and in the molecular technologies available to dissect the action of the androgen receptor (AR) are reshaping therapeutic goals. Consequently, new methods of monitoring patient hormonal status and response are needed. Unlike the immunoassays currently used in clinical settings to measure selected steroid hormones,

yeast-based bioassays are designed to assess AR response to all of the available ligands in a complex biological sample like serum.

In addition, yeast-based AR bioassays can be used to evaluate the impact of AR mutations and coactivators on the ligand response. The potential utility of such bioassays in a clinical environment is currently limited by the availability of human expertise and labor, the cost of reagents and microtiter plates, and in some cases the amount of biological sample available. Members of the MIT Bio-Instrumentation Lab have created a two-dimensional microchannel array technology, with the potential to initiate and monitor 10,000+ yeast-based assays in parallel with <100 nl/test point. With the successful fabrication and validation of this yeast AR biochip system, it will be possible to monitor changes in one of the major biological determinants of prostate cancer progression - the bioavailable levels of androgen and other AR ligands - in a timely and cost effective manner.

James G. Keck
GeneTrace Systems Inc
Improving Target Validation

James G. Keck GeneTrace Systems Inc., Alameda, CA

The pharmaceutical industry has made substantial genomics investments to help aid in the development of novel therapeutics. While these efforts have led to an abundance of structural genomic information, few validated targets and even fewer drugs have been developed to fight cancer and other diseases. GeneTrace is developing a comprehensive and integrated platform of technologies to address this bottleneck in target validation. NCI has supported and aided our progress in developing and applying these technologies to cancer research. The current status of our research program will be presented.

Alex A. Kogon
Biolinx, LLC
Thomsen-Friederich Antigen Assay for Cancer Screening

Alex A. Kogon and Elena N. Peletskaya Biolinx, LLC, Hagerstown, MD

The development of a sensitive assay for the detection of Thomsen-Friederich antigen in human body fluids is proposed. Highly specific peptides were selected through phage display libraries to target this carbohydrate antigen associated with various human tumors (Kd of peptide-antigen binding up to 5 nM). To facilitate the immobilization of the above peptides on solid support without loss of affinity, reduce nonspecific interactions in the assay, and eliminate blocking step, a new approach in modification of low sorption supports was developed using photoactivatable carbene-generating crosslinkers. Based on this immobilized-affinity-peptides setup, three different reporting

systems (ELISA-type sandwich, fluorescence quencher competition, and enzyme conjugate competition) will be tested and optimized for the highest T-antigen signal-to-background ratio. The resulting dipstick-type test kit is expected to combine high performance with one-step simplicity. We believe this development will provide an inexpensive and reliable method of noninvasive early stage cancer screening and detection.

Robert W. Kwiatkowski
Third Wave Technologies, Inc

The Invader(r) Technology for Genotyping and Quantitative Analysis of Nucleic Acids on Solid Supports

Bruce Neri[1], Victor Lyamichev[1], Jeff Hall[1], Robert W. Kwiatkowski[1], Priscilla Wilkins Stevens[2], David Kelso[2], Manchun Lu[3], and Lloyd Smith[3] [1]Third Wave Technologies, Inc., Madison, WI, [2]Northwestern University, Evanston, IL and [3]University of Wisconsin, Madison, WI

The Invader technology has been developed for the detection of nucleic acids. It is a signal amplification system that is able to accurately quantify DNA and RNA targets with high sensitivity. Exquisite specificity is achieved by combining hybridization with enzyme recognition, which provides the ability to discriminate mutant from wild-type at ratios of greater than 1/1000 (mt/wt). The technology is isothermal and flexible, and it incorporates a homogeneous fluorescence readout. It is therefore readily adaptable for use in clinical reference labs as well as in high-throughput applications using 96-, 384- and 1536-well formats with either endpoint or real time detection. The goal of this research is to adapt the technology for use on solid supports which will permit massively parallel, ultra high-throughput screening of single nucleotide polymorphisms (SNPs) and changes in gene expression. Combining signal amplification and high specificity with the ability to amplify signal at a discrete, addressable location on a solid support will enable multiple assays to be performed on a single sample, without the need for prior target amplification. This will enable the development of a high-throughput, low-cost system that is capable of determining the significance of genomic changes and providing the information needed to link genetics to the management of therapy, thereby achieving the goal of personalized medicine.

Eric Lader
Ambion, Inc

Optimization of RNA Recovery from Fixed Tissue Samples

Eric Lader Ambion, Inc, Austin, TX

A variety of molecular pathological procedures involving RT-PCR are rapidly evolving from research to the clinical laboratory. Routine use of RNA from preserved cell and tissue samples for molecular diagnostics will depend on improving the methods used to

preserve and isolate intact RNA. Currently, the recovery of high-quality RNA from neutral buffered formalin (NBF) preserved clinical samples is not possible. The RNA isolated from NBF-fixed tissues is recovered in extremely low yield, is degraded, and suffers from poor transcriptional competence. The most critical limitation we wish to address is the poor performance of recovered RNA as template for reverse transcription. Although it is rarely discussed in the literature, chemical modifications that occur to RNA during fixation render it a poor template for reverse transcription. Regardless of the size of the recovered RNA, only small stretches can be copied into cDNA or amplified by PCR.

Our goal is to develop reagents and procedures to optimize the recovery and reverse transcription competency of RNA isolated from traditional formaldehyde-fixed samples. Our efforts will concentrate on improving the recovery of RNA, minimizing degradation, and reversing the NBF-induced modifications that occur during fixation. Other techniques that depend upon the transcriptional competence of RNA, such as *in situ* RT-PCR, should also benefit from these improvements. A second goal of this proposal is to perform a systematic evaluation of alternative crosslinking and fixation reagents in order to identify one that allows superior RNA recovery and still provides acceptable histology for a broad range of tissue types.

Nadia Lifshitz
BioPhotonics Corporation

High-Resolution Sequencing of a Mixed Population of DNA Molecules

Nadia Lifshitz[1], Boris. Gorbovitski[1], and Vera Gorfinkel[2] [1]BioPhotonics Corporation, Pittsfield, MA, [2]ECE Department, State University of New York, Stony Brook, NY

We have designed and implemented a high-performance single capillary DNA sequencing instrument that possesses expanded dynamic range (23 bits), high sensitivity, and low signal-to-noise ratio.

The instrument operation is based on the novel multicolor detection technique for implementation in DNA sequencing, which is based on the illumination of the sample by several different light sources, each with a different wavelength pi, and modulated in time in a distinguishable way. The resulting fluorescence is detected by a single photon counter. The instrument is equipped with a special injection visualizer that consists of a laser connected to an optical fiber, with the tip fixed to a micropositioner, and a CCD camera connected to a computer. When the laser beam illuminates dye-labeled DNA fragments, the excited fluorescence can be directly recorded and displayed. Through the window in the capillary coating at the inlet, we can observe in real time the process of the electrokinetic injection and the initial zone separation.

With this instrument, we have demonstrated that it is possible to detect small amounts (down to 1%) of genetic material on the background of the host material using capillary DNA electrophoresis. This implies that our instrument will be capable of detecting a

mutant base against a background of wild-type bases at a ratio of 1 in 100. This unique property makes this instrument specifically suitable for analysis of mixed DNA samples with low present mutations, a task that is beyond the ability of the existing DNA sequencing instruments.

Paul M. Lizardi
Department of Pathology, Yale University School of Medicine
Messenger RNA Profiling by Single Molecule Counting

Stefan Hamann[1], John Leamon[1], David C. Ward2, and Paul M. Lizardi[1] [1]Department of Pathology and [2]Department of Genetics, Yale University School of Medicine, New Haven, CT

The aim of this project is to develop reliable protocols that facilitate the analysis of gene expression profiles in very small tissue samples. We have implemented a two-step approach designed to minimize the risk of introducing amplification artifacts. In the first step, cDNA is subjected to a step of limited linear amplification, using *in vitro* transcription (Eberwine at al., 1992, PNAS 89:3010-14). This single step amplifies the cDNA approximately 500-fold. Amplified RNA is then converted to single-stranded DNA by random priming with two different primers (control and tester). After hybridization on standard cDNA microarrays, the slides are washed and subjected to surface-anchored rolling circle amplification (RCA-CACHET, Lizardi et al., Nature Genetics 19:225-32), which amplifies two different primers (control and tester) connected by tethering to the specifically bound cDNA. Fluorescent oligonucleotide decorators (labeled with Cy3 and Cy5) are bound specifically to the two types of amplified DNA generated during RCA. This step provides approximately 60-fold signal amplification in single-primer RCA mode, and 300-fold amplification using a modified two-primer RCA mode. Amplified signals are analyzed using a high-resolution microarray reader capable of counting signals arising from single molecules of amplified DNA in the lowest intensity spots of the microarray. We are now at the stage of evaluating if the expression data generated using the RCA method is free from amplification-induced distortions.

Robert Lucito and Michael Wigler
Cold Spring Harbor Laboratory
Detecting Gene Copy Number Fluctuations in Tumor Cells by Microarray

Robert Lucito1, Joseph West[1], Andrew Reiner[1], Joan Alexander[1], Diane Esposito[1], Bhubaneswar Mishra[2], Scott Powers[3], Larry Norton[4], and Michael Wigler[1] [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Courant Institute, New York University, New York, NY, [3]Tularik, Greenlawn, NY, [4]Memorial Sloan-Kettering, Division of Solid Tumor Oncology, New York, NY

We have explored the use of representations in conjunction with DNA microarray technology to measure gene copy number changes in cancer. We have demonstrated

that arrays of DNA probes derived from low-complexity representations can be used to detect both amplifications and deletions when hybridized to representations of genomic DNA. The method is both reproducible and verifiable, and it is applicable even to microscopic amounts of primary tumors. We also present a mathematical model for array performance that is useful for designing and understanding DNA microarray hybridization protocols. The future applications and challenges of this approach are discussed.

Robert H. Mach
Departments of Radiology and Physiology and Pharmacology, Wake Forest University School of Medicine

Sigma Receptors as a Biomarker of the Proliferative Status of Solid Tumors

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Radionuclide imaging procedures such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) are having an increasing role in the diagnosis and therapeutic monitoring of cancer patients. Unlike techniques such as DNA flow cytometry of biopsy samples or fine needle aspirates, which sample only a small fraction of the tumor, SPECT and PET can image and provide information about the entire tumor. A relatively new approach toward developing radiotracers for imaging tumors is the use of radiolabeled small molecules that possess a high affinity for receptors that are abnormally expressed in tumor cells. Examples of this approach in breast cancer imaging include the use of radiolabeled estrogen and somatostatin analogues that determine the estrogen- and somatostatin-receptor status of a tumor. The goal of these imaging studies has been to predict the potential success of antiestrogens or somatostatin agonists in the treatment of cancer.

One of the goals of our research program over the past five years has been to identify a receptor that could serve as a potential biomarker of the proliferative status of solid tumors. We initially focused on sigma receptors because a number of studies have reported an overexpression of these receptors in a variety of human tumors. Sigma receptors represent a class of proteins that were originally, and falsely, classified as a subtype of the opiate receptors. Subsequent studies revealed that sigma binding sites represent a distinct class of receptors that are located in the central nervous system as well as in a variety of tissues and organs. Radioligand binding studies and biochemical analyses have shown that there are two types of sigma receptors, termed S1 and S2. Using a well-established mouse mammary adenocarcinoma cell line (66) we recently demonstrated that:

- the density of S2 receptors is higher in proliferative mouse mammary adenocarcinoma cells versus that of nonproliferative or quiescent mouse mammary adenocarcinoma cells (Mach et al., Cancer Research 57: 156; 1997.)
- the upregulation and downregulation of this receptor follows the kinetics of mouse mammary adenocarcinoma cells entering and exiting the cell cycle. The kinetics

of the up- and downregulation of the S2 receptor was found to correspond to that of PCNA, a known marker of tumor cell proliferation (Al-Nabulsi et al., British Journal of Cancer 81: 925; 1999).

- the 10-fold difference in the S2 receptor density between proliferative and quiescent mouse mammary adenocarcinoma cells grown in tissue culture also occurs in solid tumors growing in nude mice (Wheeler et al., British Journal of Cancer 82: 1223; 2000).

These data suggest that S2 receptors are potential biomarkers of cell proliferation in solid tumors, including breast, sarcomas, melanoma, lung, and head and neck tumors. Preclinical imaging studies conducted in our laboratory indicate that s2-selective radiotracers provide excellent images of solid tumor xenographs growing in nude mice. These data support the concept of using S receptor-based radiotracers for assessing the proliferative status of solid tumors *in vivo* with radionuclide imaging techniques such as PET and SPECT.

Arun Majumdar
Department of Mechanical Engineering, University of California

Optomechanical Detection of Biomolecular Recognition and Interactions

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Molecular diagnosis is increasingly being used for detecting infectious and genetic as well as neoplastic diseases. The detection of disease-related molecules is often achieved by virtue of binding of the target molecule to its specific ligand. There are three major modalities for recognition of intermolecular binding: (a) *in situ* assays (e.g. *in situ* hybridization, immunohistochemistry, *in situ* PCR); (b) solid matrix-based reactions (e.g. using nitrocellulose/nylon membranes, microwell plates); and (c) solution assays (e.g. solution hybridization, PCR). While increasingly sensitive labels for analyses are being developed, these techniques are either expensive or limited to detecting only a few types of molecules at a time. DNA microarray chips, on the other hand, can simultaneously detect a large number of nucleic acid sequences and are now increasingly being used for high-throughput genomic analysis. Because of fluorescence detection, however, these chips require laser and imaging optics and either on-chip or external electronics. In addition, they are limited to only nucleic acid analysis.

Here we present a new approach of optomechanically detecting biomolecular binding. We show that when binding between biological molecules is confined to one surface of a microcantilever beam, the resulting differential change in surface free energy density produces sufficient force to bend the cantilever. Using self-assembled monolayers of single-stranded DNA on microcantilever beams, DNA hybridization reactions can be

mechanically detected. This technique is sufficiently sensitive to distinguish oligonucleotide sequences differing in length by a single nucleotide. Using thermodynamic arguments and the mechanical detection of biotin-neutravidin binding as an example, we propose that this approach is sufficiently general to detect a potentially wide range of biomolecular interactions, including antigen-antibody binding. The possibility of mechanically detecting biomolecules without the use of fluorophores or other labels offers the promising prospect of developing micromechanical arrays for high-throughput genomic and proteomic analysis.

G. Mike Makrigiorgos
Dana Farber Cancer Institute and Harvard Medical School

Highly Selective Isolation of Unknown Mutations in Diverse DNA Fragments: New Potential for Multiplex Screening in Cancer

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The lack of methods that allow simultaneous screening of several unknown gene mutations currently hampers the large-scale detection of genetic alterations in complex DNA samples. Here, we present the feasibility of highly efficient isolation and amplification of DNA containing unknown mutations out of a mixture of unknown, diverse nucleic acid fragments, using a novel mismatch-capture methodology (A.L.B.U.M.S: Aldehyde linker-based ultrasensitive mismatch scanning, Cancer Research 2000, In Press). Heteroduplexes are formed via cross-hybridization of mutation-bearing and normal DNA. Aldehydes are uniquely introduced at mismatched positions via treatment with glycosylases, then covalently linked to a biotinylated hydroxylamine. Biotinylated DNA fragments are ligated to synthetic linkers, separated from non-biotinylated fragments, and PCR-amplified. Mutation-bearing fragments can be subsequently cloned, sequenced, or applied on microarrays. The method correctly detects a single T-to-G transversion introduced into a 7,091 base pair DNA fragment, even in the presence of a high excess (1:1000) wild type: mutant plasmids, or in the presence of diverse DNA fragment sizes. A.L.B.U.M.S extends the current limit for unknown mutation scanning by 2-3 orders of magnitude (1 mutation in 7 Mega-Bases DNA) and allows large-scale mutation scanning in complex DNA. Supported by NIH grants CA 69296 and CA 83234.

Sergei Malkhosyan
The Burnham Institute

Comparative Hybridization of AP-PCR Arrays

Sergei Malkhosyan The Burnham Institute, La Jolla, CA

We applied an unbiased DNA fingerprinting technique, the Arbitrarily-Primed PCR (AP-PCR), to study tumor-specific genetic changes. AP-PCR uses a single oligonucleotide

primer of arbitrary sequence and generates a profile of quantitative and qualitative differences between the fingerprints of tumor and matched-normal tissues. Our efforts to both automate the AP-PCR technique and make it more robust gave us the idea of combining AP-PCR fingerprinting with DNA array hybridization technology. We call this new technique Comparative Hybridization of AP-PCR Arrays (CHAPA). We propose to clone individual DNA fragments amplified from human genomic DNA by AP-PCR and array them on a solid base.

The array will be hybridized with AP-PCR products that are amplified from normal and tumor tissue DNAs, which have been labeled with green and red fluorescent dyes, respectively. The intensity ratio of the two colors at each hybridization spot will reflect tumor-specific losses or gains at corresponding genomic loci. Our plan is to develop a small array of 300 AP-PCR fragments and test the new technique to see if it is reproducible, sensitive, and reliable. Subchromosomal origins of the arrayed AP-PCR fragments will be determined by hybridizing the array with AP-PCR products from individual clones of radiation hybrid mapping panels. We will test different arbitrary primers to obtain collections of AP-PCR products of the human genome with a degree of complexity that is optimal for a large scale array of AP-PCR fragments. We will also test AP-PCR's ability to produce quantitative fingerprints of genomic DNA isolated from minute amounts of fixed microdissected tissues. In its final form, the technique will allow the state of the tumor genome to be quickly and automatically analyzed with less than 1 Mb of resolution. This high-density unbiased molecular karyotyping will facilitate the discovery of novel cancer genes and open new horizons for the diagnostic and prognostic analysis of cancer development.

Michael McClelland
Sidney Kimmel Cancer Center

Rare Transcript Detection in Microarray Analysis

John Welsh, Thomas Trenkle, Steve Ringquist, and Michael McClelland Sidney Kimmel Cancer Center, San Diego, CA

We are developing methods to produce cDNA probes for microarray analysis, where the representation of a subset of rare transcripts is enhanced. One approach uses arbitrarily primed PCR. We have demonstrated that the products of RNA arbitrarily primed (RAP-PCR) are selected primarily based on their match with the primer, rather than their abundance. This leads to a bias toward the low-abundance class because the low-abundance class contains the vast majority of the complexity of the mRNA population. Thus, the RAP-PCR probes have an entirely different spectrum of abundances than the original mRNA population. For example, on an array of 18,000 ESTs, less than 5% of the top 2000 ESTs detected by a randomly primed cDNA probe derived from a cell line are among the top 2000 ESTs detected by a RAP probe derived from the same RNA.

In another experiment, Cy5-labeled randomly primed cDNA was compared to a Cy3-labeled RAP sample. The probes were hybridized to any array consisting of about 3000

ESTs from an un-normalized library and 3000 clones from a normalized library. Both probes hybridized to a similar proportion of clones in an un-normalized EST array. However, the RAP probe vastly predominated in an array from a normalized library. This indicates that the RAP probe contained abundant PCR products from mRNAs that were relatively rare in the mRNA population. The RAP sample is very reproducible, and any differences in an individual mRNA species between two RNA populations is preserved when that mRNA is sampled, allowing changes in mRNA abundance to be monitored. Variability in these probes is comparable to the variability seen between two conventional cDNA probes developed by random priming. The ability of RAP probes to easily detect some rare messages occurs because the probe complexity is reduced, meaning that some mRNAs are not sampled efficiently. Thus, obtaining nearly full coverage requires more than one RAP-PCR probe to be used. Nevertheless, using such probes is likely to be of great utility because they will reveal changes in RNA transcript abundance among the rarest transcripts, which are likely to include transcripts for some important regulatory proteins.

Deirdre Meldrum
University of Washington

Automated Minimal Residual Disease Quantification

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Quantification of Minimal Residual Disease (MRD) is a general concern in oncology, since this parameter is likely to give valuable information to clinicians to allow them to customize chemotherapeutic treatments and anticipate possible relapses. An automated system will be developed for the quantification of MRD by using real-time PCR to quantify cancer cells in a background of non-pathologic DNA. A high-performance custom fluorescence analyzer will be developed with a signal-to-noise ratio and a dynamic range of at least one order of magnitude larger than the optics used on commercial systems. This unique feature will permit precise measurements of the amplification kinetics during at least five cycles in the exponential phase of the reaction. The amplification yield will be derived from these data using statistical estimators customized to meet the requirements of real-time PCR data analysis. The sample DNA content will be derived from the amplification yield and the calibrated fluorescence measurements of the reaction kinetics.

This new approach will make it possible to run a series of real-time PCRs with more flexibility than would be possible if the assay was based on standard reactions required to have the same amplification yield as the clinical samples. PCR conditions will be adapted online without concerns about possible differences of amplification rate. The assay control algorithm will adapt the reaction DNA content, the primer selection, and the number of PCRs to meet the clinician requirements for a particular patient is DNA. In the R21phase of the project, a real-time thermocycler prototype will be developed to

demonstrate the optics performance and the possibility to estimate PCR amplification rates with 5% accuracy. In the R33 phase of the project, the hardware and software components will be developed and integrated into an automated system. Performance of the system will be evaluated on real biological samples, and the results returned by the system will be compared with results of t(14:18) PCR performed with an ABI PRISM 7700 for the diagnosis of patients suffering from follicular lymphomas.

Seajin Oh
SRI International
An AFM-Based Technique for Analyzing Expression in Cancer Cells

Seajin Oh[1], William Wright[1], Keith Laderoute[1], Eric Henderson[2], and Curtis Mosher[2] [1]SRI International and [2]BioForce Laboratory, Menlo Park, CA

We propose to demonstrate and develop an AFM-based technology involving a submicron scale probe array, and, by using the array, directly monitor protein interactions from cellular lysates. This technology will allow the analysis of the constituents of cells suspected or known to be involved in oncogenesis. In the technology, AFM tips pick up protein or nucleic acid probes from reservoirs, transfer them to a support, and deposit them at selected array positions. The process is repeated to complete a protein or nucleic acid array, which has submicron space between the array positions, (e.g., a 100 × 100 array in about a 0.1 mm × 0.1 mm area). Target proteins are applied to the array, and the binding events on the arrayed molecules are monitored by AFM topography measurements.

By carrying out these processes in a miniaturized format and on very small samples, the cost of the analysis will be minimized and the rate will be maximized. Use of a multiple tip array further speeds analysis rate. The proposed research consists of two phases. In the first phase (R21), we will demonstrate the manipulation of single molecules by the AFM tip and the detection of single molecular binding events by AFM topography measurement. A molecule involved in signal transduction will be selected and monitored by this technique. In the second phase (R33), we will extend the capability of the AFM technique by simultaneously monitoring different molecules associated with parallel signaling events. The immediate purpose of these studies is to determine the relative contributions of established parallel signaling pathways to the transmission of growth signals in cancer or transformed cells. Ultimately, this technique will be used to investigate and determine the assembly of multiprotein complexes isolated from cancer cells.

Maria Pallavicini
University of California, San Francisco
Protein Signatures of Leukemic Cells

Maria Pallavicini and Alma Burlingame University of California, San Francisco, CA

The overall goal of this R21/R33 application is to develop and apply comparative genome-wide proteomic approaches to determine the extent to which protein/peptide signatures of malignant cells enhance information obtained from cytogenetic and histopathologic analyses. Specifically, we will evaluate the relationship between molecular cytogenetic aberrations and protein expression in myeloid leukemia and determine whether distinctive patterns of protein expression (e.g., protein/peptide signatures) predict treatment response. Two-dimensional (2-D) gel electrophoresis, time-of-flight mass spectrometry (MALDI-TOF-MS) and nanoliter/min flow rate electrospray mass spectrometry (ESI-MS) will be combined in a novel approach to map low and high abundance proteins/peptides in cell and nuclear lysates from primary leukemic specimens.

In the R21 application we will establish methodologies to optimize reproducible proteome sampling of clinical specimens. In the R33 application we will pursue two parallel approaches, based on 2-D gel separation of proteins according to isoelectric points (pI) and mass, to investigate the proteome of clinical leukemic specimens. In one approach, we will quantify differentially expressed moderate-to-high abundance proteins in cell and nuclei lysates from normal and leukemic specimens using sample processing and in-gel protein detection. In another approach, we will create peptide maps of the entire 2-D gel of cell and nuclear lysates from clinical specimens to increase the dynamic range of protein measurements on 2-D gels by identifying low abundance proteins. Both of these approaches will be used to 1) establish the protein signature of primary t(15;17) APL specimens at diagnosis and relapse using low and high abundance protein/peptide maps and 2) determine the extent to which protein/peptide signatures of APL at diagnosis predict treatment response. Additionally, we will initiate studies to develop antibody-based reagents to test the clinical potential of the gel protein signature associated with poor prognoses APL.

We anticipate that protein/peptide signatures will lend new insight into the physiologically active forms of proteins associated with molecular cytogenetic aberrations, increase understanding about regulators of tumor phenotypes, and identify novel diagnostic and predictive tumor markers.

Mark W. Perlin
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Automated Multiplex Genetic Analysis Technology

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Recent high-throughput multiplex genetic technologies have enabled orders-of-magnitude advances in our ability to assay gene expression and chromosomal regions. For example, high-throughput capillary electrophoresis (CE) instruments can generate 10,000 data traces per day. These advances are necessary for accelerating cancer

research. However, this ability to rapidly generate data has far outstripped our capability to review, edit, and enter such data for downstream computer analysis. A key bottleneck now impeding research progress is the (computer-assisted) manual scoring of data.

This presentation focuses on the automated scoring of such multiplex genetic data, including differential-display, microsatellite, and SNP assays. We are adapting, developing, and refining fragment analysis computer software for modern high-throughput DNA separation instruments. This software will automatically score multiplex data from both gene expression and chromosomal analysis experiments and assess their quality. It will be able to use data generated on diverse DNA sequencing instruments, and it will run on all common computer hardware. Such automation should be useful in high-throughput settings, such as searching for cancer-related genes, and (ultimately) in clinical testing.

J.M. Ramsey
Chemical and Analytical Sciences Division, Oak Ridge National Laboratory
Microfabricated Fluidic Devices for Protein and Peptide Mapping

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Miniaturized chemical instruments, or Lab-on-a-Chip technologies, are being developed for rapid, comprehensive analysis of cellular proteins as alternatives to the slow and labor-intensive 2D gel methods currently used for protein mapping. The microfabricated devices integrate on a single structure as elements that enable multidimensional separations of protein or peptide mixtures and on-chip labeling for fluorescence detection of rapidly migrating analyte bands. The platform exploits the many advantages of microfluidic devices, including small size, inexpensive fabrication, high speed, low-volume materials consumption, high throughput, and automated operation. First generation devices, combining open channel electrochromatography or micellar electrokinetic chromatography with open channel electrophoresis, have been demonstrated for the analysis of proteins and tryptic digests. Analysis times are on the order of a few minutes or minimally two orders of magnitude faster than required for conventional 2D-PAGE. Other microchip structures that incorporate an electrospray element have also been devised and interfaced to a time-of-flight mass spectrometer with acquisition rates needed to capture mass spectra from rapidly eluting components. Spectra have been obtained in only 10 ms from subattomole quantities of peptides electrosprayed from chips. Rapid on-chip enzymatic digestion has also been demonstrated and used to analyze hemoglobin variants.

Peter Rheinstein
Cell Works Inc
Isolation and Profiling of Circulating Prostate Cancer Cells in Blood

Paul Tso[1], Zheng-Pin Wang[1], Stephen Lesko[1], Michael Carducci[2] and Peter Rheinstein[1] [1]Cell Works Inc. and 2Johns Hopkins University, Baltimore, MD

The major goal of the project was to develop assays at the single cell level (viz., FISH and immunofluorescence) for producing molecular profiles that may be predictive of the metastatic potential of circulating cancer cells and thus serve as a prognostic tool. Nuclear DNA can be quantified by measuring the fluorescence of bound DAPI, a DNA-specific dye, and comparing the cancer cell DNA content with that of reference WBCs (2N DNA) on the same slide. The observed cancer cell/WBC ratios of integrated fluorescence intensity were LnCap, 95%> 2.0; TSU, 90%> 2.0; and non-proliferating prostate cell 1.0. This DNA assay for characterize circulating PC (CPC) from patients as genetically abnormal (cancerous), epithelial/prostatic cells. Whether CPC are dividing or dying can be assessed by quantitative immunofluorescence for Ki67, thymidylate synthetase, and P27 and in addition, by TUNEL staining for DNA fragmentation. These assays have been established in laboratory and clinical studies. Finally, an immunofluorescence assay and FISH have been developed for measurement of androgen receptors (AR) and AR gene copies. This research has established assays for genetic abnormalities, dividing versus dying cells abundance of AR, and mutation of AR gene copy numbers - very important profiling for characterizing CPC.

Stanley E. Shackney
MCP/Hahnemann University, Department of Human Oncology, Allegheny General Hospital

Multiparameter Analysis of Human Epithelial Tumors by Laser Scanning Cytometry[4]

Agnese A. Pollice[1], Charles A. Smith[1], Daniel L. Farkas[2], Jan F. Silverman[3], and Stanley E. Shackney[1] [1]MCP/Hahnemann University, Department of Human Oncology, Allegheny General Hospital, Pittsburgh, PA, [2]Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, [3]MCP/Hahnemann University, Department of Laboratory Medicine, Allegheny General Hospital, Pittsburgh, PA, *[4]Supported by NIH grant # CA83204*

Background: Laser scanning cytometry (LSC) is a relatively new technology for performing multiple fluorescence measurements on individual cells deposited on slides. Techniques have been developed for performing four or more measurements on individual lymphoid cells using light scatter as a triggering parameter for cell identification. However, these techniques are not suitable for the identification of fixed epithelial tumor cells, and an alternative approach is required for the analysis of such cells by LSC.

Methods: Methods for sample preparation, event triggering, and the performance of multiple LSC measurements on fixed human cells were developed using normal lymphocytes and two human breast cancer cell lines, JC-1939 and MCF-7. Results: Optimal conditions for individual cell identification by LSC were found to depend on

several factors, including the deposited cell density (cells per unit area), the dynamic range of probe fluorescence intensities, and the intracellular distribution of the fluorescent probe. Sparsely deposited cells exhibited the least cell overlap and the brightest immunofluorescent staining. A major advantage of DNA probes over a cytoplasmic immunofluorescent probe such as tubulin was that they exhibited greater fluorescence intensity within a relatively sharply demarcated nuclear region. The DNA-binding dye LDS-751 was suboptimal for quantitative DNA measurements, but useful as a triggering measurement that permits the performance of simultaneous FITC-, phycoerythrin-, and Cy-5-based measurements on each cell. A major potential advantage of LSC over flow cytometry is the high yields of analyzable cells by LSC, permitting the performance of multiple panels of multicolor measurements on each tumor.

Conclusions: A technique has been developed for performing multiple fluorescence measurements on fixed epithelial cells by LSC. Key terms: Laser scanning cytometry, multiparameter analysis, cancer, immunofluorescence, LDS-751

A. Dean Sherry
Department of Chemistry, University of Texas at Dallas, Richardson, TX, and
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New Functional MR Agents Based on Lanthanide Complexes Displaying Slow Water Exchange

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All current Gd^{3+}-based MRI agents rely upon rapid exchange of a metal ion-bound water molecule with bulk water. Recent kinetic studies have shown that the bound water lifetimes (tM^{298}) can range from 0.84 ns for $Gdaq^{3+}$, 200-303 ns for $GdDTPA^{2-}$ and $GdDOTA^{-}$, to 2-20s for the corresponding bis- and tetra-amide derivatives. We recently reported that the water proton relaxivity (R1) of Gd (1) is unusually high for a low molecular weight complex and is pH-sensitive. This effect has been traced to protonation of the extended phosphonate groups of Gd (1), which are hydrogen-bonded to a single slowly exchanging (tM^{298}=20s) Gd^{3+}-bound water molecule.

The goal of this work is to take advantage of these unique features in designing new biologically responsive MRI agents. Interestingly, lanthanide complexes of the diethyl ester of Gd(1) display even slower water exchange kinetics (tM^{298}~400 s) in both water and acetonitrile as solvent and that the water relaxivity of these Gd^{3+} complexes is enhanced by the addition of exogenous anions. This result supports our hypothesis that the relaxivity of these complexes is governed by water proton exchange, not molecular exchange. High resolution NMR spectra of the Eu^{3+} complexes of the same ester ligands show separate ^{1}H and ^{17}O resonances for Eu^{3+}-bound and bulk water. Saturation of the highly-shifted bound water resonance (60 ppm) results in an 80% decrease in

intensity of the bulk water resonance. This feature offers the potential of a new class of magnetization transfer contrast agents that have a much higher response to biological function that conventional MRI agents.

Robert H. Singer
Departments of Anatomy and Structural Biology and Cell Biology, Albert Einstein College of Medicine
Fish & Chips

Robert H. Singer, Jeff M. Levsky, Amy R. Kurland, Steve J. Braut, and Shailesh M. Shenoy Departments of Anatomy and Structural Biology and Cell Biology, Albert Einstein College of Medicine, Bronx, NY

We introduce a new technology to allow simultaneous detection of multiple distinct transcripts by fluorescence *in situ* hybridization (FISH). Oligomer probes to mRNA sequences are prepared and labeled with different fluorescent dye combinations known as barcodes. The theoretical number of unique species that can be discerned is nearly limitless, allowing for the analysis of gene expression *in situ* for chip microarray-sized populations of genes. This method represents a significant improvement on FISH technology by permitting analysis of coordinate expression patterns, functional genomics, and nuclear organization of transcription sites. Whereas microarrays provide information about mean expression values, this assay yields cell-specific expression intensities without loss of tissue architecture. Since the tissue is undisturbed, we can apply the technology to the analysis of developing tissues, finely demarcated areas of expression, and heterogeneous tissue sections. In essence, the method unifies microarray expression studies and FISH by drawing from the strengths of both. We intend to survey genes significant in oncogenesis to produce a tool for pathologists to determine cancer characteristics and therapeutic modalities on biopsy specimens.

Paul L. Skipper[1] and Barbara J. Hughey[2]
[1]Massachusetts Institute of Technology and [2]Newton Scientific, Inc
Accelerator Mass Spectrometry of 3H for Analysis of Cancer Biomarkers

Paul L. Skipper[1], Barbara J. Hughey[2], John S. Wishnok[1], Steven R. Tannenbaum[1], Ruth E. Shefer[2], and Robert E. Klinkowstein[2] [1]Massachusetts Institute of Technology and [2]Newton Scientific, Inc, Cambridge, MA

The goal of this project is to develop an exceptionally compact, high-throughput accelerator mass spectrometer (AMS) to enable ultra-sensitive quantitation of tritium in a variety of applications related to the analysis of cancer biomarkers. AMS is a powerful tool detecting rare isotopes, such as tritium, that are commonly used to radiolabel organic biomolecules, with detection limits in the attomole (10-18 mole) and lower range. In contrast to existing instrumentation, an AMS designed exclusively for detecting tritium may be as compact and inexpensive as conventional mass

spectrometers because it will operate at much lower energy than existing multi-isotope AMS instruments. As currently practiced, analysis of biological samples by AMS is limited by the requirement for highly specialized sample preparation procedures that are not compatible with online and rapid detection applications. The proposed instrument addresses this shortcoming by integrating a specialized sample interface into the overall design to facilitate coupling with various liquid micro-flow sample preparation systems.

Richard D. Smith
Pacific Northwest National Laboratory

Advances in Proteome-Wide Protein and Functional Analysis

Richard D. Smith, Ljiljana Pasa Tolic, Gordon A. Anderson, Mary S. Lipton, Thomas P. Conrads, Timothy D. Veenstra, and Yufeng Shen Pacific Northwest National Laboratory, Richland, WA

The patterns of gene expression and protein posttranslational modifications and how these may be affected by changes in the environment, disease state, cell cycle, etc., cannot be accurately predicted from DNA sequences. Present approaches for proteome characterization are based upon mass spectrometric analysis of in-gel digested electrophoretically separated proteins, allowing relatively rapid protein identification compared to conventional approaches. However, this technique remains constrained by the speed of the 2D-PAGE separations, the sensitivity needed for protein visualization, the speed and sensitivity of subsequent mass spectrometric analyses for identification, and the limitations of protein "spot" visualization for quantitation.

Our objective is to develop and apply an alternative approach for broad proteome analysis based upon the combination of fast high resolution capillary electrophoresis or chromatographic separations and the high accuracy of mass measurements and sensitivity obtainable with Fourier transform ion cyclotron resonance mass spectrometry. Protein identification is based upon global approaches for protein digestion and accurate peptide mass analysis for the generation of Accurate Mass Tags. In the same analysis, precise measurements of relative protein expression are obtained by the inclusion of a stable-isotope labeled "reference proteome", enabling measurements of protein expression with precisions of better than 10% and sensitivities in the attomole range. In conjunction with Prof. Ruedi Aebersold (U. Washington), we are also exploring the use of an alternative labeing technology that both reduces the complexity of the peptide mixture and provides improved protein identification. This technology is anticipated to enable the analysis of mammalian proteomes. The status of efforts towards the development of a high throughput proteomics capability, initial results for application to several microorganisms, and preliminary results for extension to mammalian proteomes will be presented.

Martin Stanton
Brandeis University

Aptamer-Based Detector for Quantification of Biological Macromolecules

Martin Stanton[1] and Andrew Ellington[2] [1]Brandeis University, Waltham, MA and [2]University of Texas, Austin, TX

We are developing technology to simultaneously detect and quantify large numbers of individual proteins and posttranslationally modified proteins. This technology is based on a spatially-arrayed nucleic acid microchip and sensitive fluorescence-based optical detection techniques. The system will provide a powerful new tool for biomedical research and diagnosis of diseases and cancers.

Currently, levels of cellular proteins are inferred from cellular mRNA levels by using DNA oligonucleotide microchips. These microchips allow the detection of mRNA levels by basepairing interactions to homologous DNA oligomers located at different sites in the microchip array. However, this indirect assay of protein levels suffers from two problems: First, mRNA levels may not accurately reflect the level of encoded protein because translation and degradation rates vary from protein to protein and are regulated in response to cell physiology. Second, posttranslational modifications proteins often alter critical protein activities.

The technology we are developing is comprised of three parts: 1) Nucleic acid sensor molecules with high affinity and high specificity for individual proteins, designed to have ligand-dependent changes in fluorescence properties. 2) A microchip array with each array site occupied by nucleic acids against a different target molecule. 3) An evanescent field fluorescence detector which detects target molecules binding to nucleic acids at the array sites. Our project includes plans for: 1) selection of nucleic acid sensor molecules that can discriminate between different posttranslational modifications of the same protein; 2) development of fluorescence-based techniques for detecting nucleic acid:protein interactions; 3) design and construction of an optimized fluorescence detector; 4) scaling up methods to allow large numbers of independent nucleic acid selections against different proteins; 5) construction of a prototype protein chip.

Marilyn J. Stapleton
Gene Tec Corporation

Analyzing RNA Relative to DNA Directly in Minute Specimens

Marilyn J. Stapleton and Ke Wei Gene Tec Corporation

Immobilized sample amplification, or ISA, is based upon amplifying DNA and RNA directly from specimens without prior isolation of the nucleic acids. In ISA, the native RNA to DNA ratio in each sample is unperturbed. ISA devices and techniques were developed to collect a variety of liquid and solid specimens such as tissue culture cells, human tumors, whole blood, and cheek cells. Fresh specimens are dried on ISA matrices, washed, and incorporated into an amplification reaction. The amount of tissue

imprinted for ISA analysis is much less than what is needed for extraction procedures. Thus, much smaller and multiple imprints are feasible in capturing snapshots of active RNA transcription.

The new approach amplifies and measures the relative ratio of RNA to DNA in the same specimen with fewer manipulations than extraction procedures and effectively reduces random variation. Gene expression can be expressed as the ratio of band intensities after gel electrophoresis of the amplified products from RNA to DNA templates. Different expression levels of HER-2 and TNF* were detected from ISA prints of malignant and benign breast tissue. Comparing the signal intensities of amplified products from the same gene in several samples normalizes cell numbers among the samples.

William M. Strauss
Harvard Institute of Human Genetics, Beth Israel Deaconess Medical Center

Whole Genome Analysis: Towards Single Molecule Detection of Aneuploidies, LOH, and Functional LOH

William M. Strauss Harvard Institute of Human Genetics, Beth Israel Deaconess Medical Center, Boston, MA

Our goals are to develop technological infrastructure to permit the precise definition of chromosome number, gene dose, and functional gene dose in single cells while preserving the three-dimensional architecture of the nuclear environment or the covalent structure of the chromosome. To these ends, we have embarked on experiments directed at determining genome composition in single cells by developing the potential of PNA-FISH.

We have extended our earlier work to show that individual 14-20mer peptide nucleic acid (PNA) probes directed against interspersed a-satellite sequences can specifically identify chromosomes and chromosome number. PNA probes were used to detect chromosomal abnormalities and repeat structure in the human genome using fluorescence insitu hybridization (FISH). The hybridization of a single PNA probe directed against a highly abundant a-satellite DNA repeat sequence was sufficient to absolutely identify a chromosome. Ten chromosome-specific PNA probes for human chromosomes 1, 2, 7, 9, 11, 17, 18, X, and Y have been identified. Interphase and metaphase results demonstrate that chromosome specific PNA probes are capable of detecting simple aneuploidies (trisomies) in human. Another set of PNA probes showed distinct banding-like patterns and could be used as a set of sequence-specific stains for chromosome bar coding.

Another major goal for our group is the definition of technology to identify and quantify single copy loci. If successful, this technology would permit the measurement of gene dose in the context of the chromosomal environment. Recent technical innovations have yielded results that suggest that single locus testing is achievable in single cells on single molecules by PNA-FISH.

Mary N. Teruel
Department of Molecular Pharmacology, Stanford University
Crosstalk between Lipid Second Messengers in Signaling Pathways

Mary N. Teruel and Tobias Meyer Department of Molecular Pharmacology, Stanford University, Stanford, CA

There is more and more evidence for the existence of crosstalk and feedback loops in signaling pathways, particularly in those involved in growth and differentiation, where several thousand gene products may be involved. Signaling pathways can no longer be thought of as independent, linear sets of events, but rather they must be thought of as dynamic networks.

Currently there are excellent assays to establish the identity of different players in the network or to obtain final readouts that reveal which genes are activated after stimulation. However, there is a lack of tools with which to study networks dynamically and to understand how the players functionally interact in the context of a receptor stimulus. An appropriate tool for probing signaling networks should have the following features: 1) capability to simultaneously measure time courses of multiple signaling steps at the single cell level and 2) capability to obtain large numbers of single cell measurements. We have recently developed such a tool called E-SCAT, or Evanescent-wave Single Cell Array Technology, and have shown that we can measure rapid time courses of protein activation in thousands of single cells simultaneously. By developing this E-SCAT system, along with methodology to perturb the network it is monitoring, we will establish quantitative kinetic relationships in signaling networks and will be able to define the strength of crosstalk between second messenger signaling systems. Initial model signaling cascades being studied include the cascades triggered by PDGF-stimulation of NIH-3T3 fibroblast cells and those stimulated by antigen-stimulation of RBL cells, a tumor mast cell line.

Overall, this project will merge functional genomics with new assays to measure time courses and amplitudes of signaling events in single cells, with the goal of providing important insights into the quantitative and dynamic parameters of signaling networks. The technology development and implementation that is part of the project will also provide a new tool for cell-based drug discovery.

Marc Vidal
Dana-Farber Cancer Institute and Department of Genetics, Harvard Medical School
The C. elegans ORFeome Cloning Project

Jerome Reboul[1], Philippe Vaglio[1], Lisa Matthews[1], Cindy Jackson[2], Troy Moore[2], Jean Thierry-Mieg[3], Danielle Thierry-Mieg[3], Gary Temple[4], Mike Brasch[4], Jim Hartley4, Nia Tzellas[1] and Marc Vidal[1] [1]Dana-Farber Cancer Institute and Department of Genetics, Harvard Medical School, Boston, MA, [2]Research Genetics, Huntsville, AL, [3]IGM-CNRS, Montpellier, France, and [4]Life Technologies Inc., Rockville, MD

In addition to gene-based functional genomics approaches such as large-scale gene knockouts and microarray or chip analyses, it is also important to develop genome-wide protein-based approaches, e.g. protein interaction mapping, protein localization mapping, and biochemical and structural genomics. Two strategies have been developed for the characterization of (nearly) complete sets of proteins (proteomes). In the bottom-up strategy, endogeneous proteins expressed *in vivo* are analyzed using high-throughput techniques such as mass spectrometry (MS) or matrix-laser desorption/ionization (MALDI). In the top-down strategy, (nearly) complete sets of ORFs (ORFeomes) are expressed exogeneously for various protein function assays such as two-hybrid analyses, biochemical assays, protein production and purification for structural analyses, etc. The top-down approach relies upon the availability of ORFeomes cloned into various expression vectors. Our laboratory is currently cloning the C. elegans ORFeome using GatewayTM, a recombination cloning technique (RC). This system allows both the initial cloning of ORFs and their subsequent transfer into different expression vectors by site-specific recombination *in vitro* (Walhout et al., 2000, Science, 287, 166-122). The features of RC make it amenable to automation which is crucial for large-scale ORFeome cloning. So far we have cloned ~1,000 C. elegans ORFs. With our current throughput, 3,000 ORFs (15% of the ORFeome) should be cloned by the time of this meeting. We will present: i) the details of RC, ii) our current throughput, iii) the cloning quality, iv) how this resource will be made available to the community, and v) how this work will help numerous functional genomics projects. The C. elegans ORFeome cloning project is likely to illustrate how to undertake ORFeome cloning projects for more complex multicellular organisms.

Yue Wang[1] and Robert Clarke[3]
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Intelligent Bioinformatics Approaches to Microarray Expression Analysis

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Visual exploration has proven to be a powerful tool for multivariate data mining and knowledge discovery. Most visualization algorithms aim to find a projection from the data space down to a visually perceivable rendering space. To reveal all of the interesting aspects of multimodal data sets living in a high-dimensional space, we introduce a hierarchical visualization algorithm that allows the complete data set to be visualized at the top level, with clusters and subclusters of data points visualized at deeper levels. The methods involve hierarchical use of standard finite normal mixtures and probabilistic principal component projections, whose parameters are estimated using the expectation-maximization and principal component neural networks under the information theoretic criteria. We demonstrate the principle of the approach on several multimodal numerical data sets, and we then apply the method to gene selection,

cluster discovery, and class prediction in microarray expression analysis for breast cancer classification.

Peter Wiktor
Engineering Arts
Piezo-Electric Pipetting Technology for DNA Analysis

Peter Wiktor[1] and Helmut Zarbl[2] [1]Engineering Arts and [2]Fred Hutchinson Cancer Research Institute, Mercer Island, WA

This grant brings together two innovative technologies: a novel fluid transfer device and a novel technique for detecting single nucleotide polymorphisms (SNPs) on cDNA arrays. Integrated together, these two technologies will have an immediate and significant impact on the molecular analysis of cancer. Actuated by piezo-electric elements, the fluid transfer technology enables the pipetting of sub-nanoliter volumes of fluid. Additionally, novel sensing technology allows the operational state of the device to be continuously monitored. The sensing technology is indispensable for massively parallel DNA analysis applications requiring thousands of different samples to be reliably aspirated and dispensed. The ultimate goal is to integrate the fluid transfer and sensor technology into an automated pipetting instrument for cancer research and general laboratory applications. Initially, the instrument will be applied to a novel technology, making it possible for the first time to detect SNPs, deletions and insertions on readily available and relatively inexpensive spotted cDNA arrays.

Robert A. Wind
Pacific Northwest National Laboratory
Integrated Optical/Magnetic Resonance Microscopy for Cellular Research

Robert A. Wind, Gary R. Holtom, Kevin R. Minard, and Brian D. Thrall Pacific Northwest National Laboratory, Richland, WA

An integrated optical (confocal)/magnetic resonance (OM/MR) microscope is being developed for studying single layers of heterogeneously populated live mammalian cells. This combined microscope will significantly improve the individual capabilities associated with each methodology by making it possible to quickly correlate optical and MR information obtained at the cellular level. In this presentation, progress on the design of the combined microscope will be reported, and two technical applications will be described. A special MR surface coil has been designed that provides optimal sensitivity in a cell layer and makes it possible to maintain the layer in a horizontal plane. The latter property is important, as it simplifies the design of the optical part of the combined microscope considerably. The MR sensitivity of this coil has been evaluated both theoretically and experimentally, with excellent agreement. Moreover, the coil has been used to investigate SK-N-SH neuroblastoma cells and JB6 mouse skin epithelial cells. It was found that it is possible to obtain MR images of these cells

with a spatial resolution of 30x30 im2 and proton MR spectra of as little as 2,000 cells in a time of 10-30 minutes. The two applications that will be discussed are the use of combined microscopy for 1) improving the spatial resolution and contrast in MR images of cellular systems, and 2) distinguishing the MR properties of different cell populations in a heterogeneous mixture.

John R. Yates
Department of Cell Biology, The Scripps Research Institute
Identification of Proteins in Complex Mixtures

John R. Yates, Dirk Wolters, and Mike Washburne Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

A recent approach to identifying the components of protein mixtures is direct analysis of the proteolytically digested proteins using liquid separation techniques and tandem mass spectrometry/database searching. As peptide mixtures become more complex, better separation techniques are required to resolve the peptide components for tandem mass spectrometry. We have examined several strategies to separate complex peptide mixtures using microcolumn LC/LC in conjunction with tandem mass spectrometry. We are developing this approach to identify proteins expressed in cells. Results from these experiments will be discussed.

Maciej Zborowski
Lerner Research Institute of The Cleveland Clinic Foundation
Rare Cancer Cell Isolation by Magnetic Deposition on Microscopic Glass Slides

Maciej Zborowski[1], Bingbing Fang[2], Lee R. Moore[1], and Jeffrey J. Chalmers[3] [1]Lerner Research Institute of The Cleveland Clinic Foundation, Cleveland, OH, [2]Case Western Reserve University, Cleveland, OH, and [3]Ohio State University, Columbus, OH

The presence of malignant breast cancer cells in bone marrow or peripheral blood is an important diagnostic factor. We tested the capacity of a novel magnetic cell analyzer developed in-house to detect rare cancer cells in mixtures with human peripheral leukocytes. Human peripheral leukocytes were spiked with cells of the MCF-7 breast carcinoma line, and the cell mixture was labeled with anti-epithelial membrane antigen antibody and a magnetic colloid. The MCF-7 cells were selectively captured on a magnetic deposition substrate (a microscopic glass slide) from the flowing cell mixture. The recovery of the MCF-7 cells from the original mixture ranged from 20% to 60%. The limit of detection of the MCF-7 cells was 1 in 106 of total cells in the mixture (n = 9). The morphology of the captured cancer cells was well preserved and comparable to that observed in cytospin smears. All deposited cells were located in a small area of 1.4 - 6 mm and could be quickly identified with an optical microscope following routine cytological staining (Wright's). This was a proof-of-principle study using a well-defined model of rare cancer cells. The clinical relevance of this study remains to be tested in

the patients' bone marrow and blood samples using antibody cocktails against the breast carcinoma cells.