2nd Principal Investigators Meeting Abstracts

2nd Principal Investigators Meeting
Innovative Molecular Analysis Technologies Programs
June 27-29, 2001
Ritz-Carlton
Washington, DC

Abstracts

Donna Albertson
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Genome-wide Measurement of DNA Copy Number on BAC Microarrays

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We have assembled arrays of genomic clones for analysis of DNA copy number alterations by comparative genomic hybridization (CGH). Relative copy number is measured at these specific loci by hybridization of fluorescently labeled test and reference DNAs as in conventional CGH. In array CGH, the resolution is determined by the genomic spacing of the target clones. Currently, we are printing arrays of > 2500 BAC clones for the human genome and 1000 clones for the mouse. Each BAC contains an STS and has been verified to be single copy by FISH mapping. Copy number abnormalities can be readily linked to the physical map and the genome sequence by the sequence tags. The use of BACs provides sufficiently bright signals so ratio variation among the clones in a comparison 10‰. We have validated the capability of?of two normal genomic DNA samples is these arrays to quantitatively distinguish between heterozygous and homozygous deletions and trisomies by analysis of cell lines and patient samples containing known gains or losses involving one or more chromosomes. High level amplifications can also be quantitatively measured on the same arrays. This high level of measurement precision and sensitivity has been achieved both through the development of procedures for preparation of BAC DNA for spotting and the development of high density printing technology. The arrays are currently being used for mapping copy number alterations occurring in cancers, developmental disorders and for cross species comparisons.

Bryan Alexander
University of New Mexico
Molecular Stating of Human Squamous Cell Skin Cancer by cDNA

Bryan Alexander, Greg Tafoya and James Gale (P.I.)

University of New Mexico

The lifetime risk for developing skin cancer is currently 1 in 5 for the USA. While only 2-5% of non-melanoma skin cancers (basal and squamous cell) become metastatic and potentially life threatening, they account for substantial morbidity and health-care expenditures. Our understanding of the molecular pathology and critical gene-networks involved in the development and progression of non-melanoma skin cancers is incomplete, resulting in poor markers for progression, prognosis and largely ineffective treatment for invasive stages. The broad objective of this project is to characterize the changes in gene expression in different stages of squamous cell carcinoma (SCC) and to identify different gene pathways involved. The first phase of this project will characterize UV-induced gene expression in cultured, primary human keratinocytes using cDNA microarray containing 4000 named genes. The second phase of the project will profile the gene-expression levels in the three most distinctive phases of the disease (actinic keratoses; SCC; and metastatic SCC). To more completely characterize these expression profiles, a microarray containing ~30,000 human cDNAs will be used (containing all ~7000 named genes and ~23,000 ESTs of unknown function). For control purposes, we will additionally collect biopsies from normal, uninvolved skin from both the arm (sun-exposed) and buttocks (sun-protected).Having two normal skin controls will add another dimension of analysis by allowing for the identification of genes that are responsive to sunlight and that may be involved in early stages of induction of SCC.

During this initial part of the project, our emphasis has been protocol optimization and control milestone experiments. Initially, our efforts were concentrated on optimization of protocols for RNA isolation and array hybridization/wash conditions. Within the array protocol, we experimented with many factors in order to maximally reduce background without losing signal intensities. Additionally, we tested a system developed by Ambion that was aimed at prolonging the life of a filter array (ie. increase the number of times a filter may be re-used without substantial lose of signal. By optimizing each step in the process (RNA isolation, RNA labeling, array hybridization/washing, array imaging and array stripping), we have robust system that allows for maximal analysis of our experiments. Having an optimized working protocol, we then focused on control experiments designed to measure the reproducibility, noise, and limitations of the Research Genetics' filter arrays. In brief, these control experiments include the following: 1. Probing a set of filters with the same probe to determine the reliability of intra-filer comparisons 2. Determining the lowest amount of RNA (in nanograms) that can be labeled and still produce adequate signal intensity 3. Stripping and reprobing a single filter repeatedly in order to see how many times it may be reliably re-used. In addition to finalizing the control experiments, we are in the process of generating expression profiles of keratinocytes and melanocytes at various time points following exposure to solar simulating light (SSL). Additionally, we intend to repeat the same time course on cells that have had repeated doses of SSL prior to the time course in order to see if the cells have an adapted response.

Nancy L. Allbritton
University of California
Profiling Ras-Activated Signal Transduction Pathways

Nancy L. Allbritton[1], Christopher E. Sims[1], Eric. J. Stanbridge[2],and David L. Van Vranken[3]

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The molecular analysis of the signal transduction pathways driving uncontrolled growth in tumor cells will have a dramatic impact upon cancer biology and patient care. New technologies such as those to identify the genome and proteome of cells hold great promise. However these methods do not provide direct measurements of the activity of molecules involved in signal transduction. Ultimately it is the activation state of molecules such as enzymes that control cell behavior, for example, fueling the growth of tumor cells. A new technology and biochemical assay, the Laser Micropipet System (LMS), has the potential to perform simultaneous biochemical analysis of the activation state of multiple signal transducing enzymes within a single cell. Such data will enable misregulated signaling of tumor cells to be assessed in both linear signaling pathways and in interconnected networks of signaling proteins. The goal of this research is to apply the LMS to the Ras signaling cascades which are of immense importance in both the basic and clinical investigation of cancer. The research will draw on methods from analytical chemistry to analyze, separate, and detect kinase substrates from single cells. The strengths of combinatorial chemistry and synthetic organic chemistry will be brought to bear on the development of new kinase substrates to be used as specific reporters of Ras-regulated kinase activation. Molecularly engineered tumor cell lines in which individual proteins have been selectively mutated will be used to demonstrate the capabilities of the LMS in measuring the activation of kinases in the Ras-regulated signaling cascades. The successful completion of this work will provide a new and powerful tool for basic research, drug discovery and screening, cancer classification, and potentially clinical decision making.

Cynthia Bamdad
Minerva Biotechnologies Corporation
Rapid Electronic Detection of Cell Surface Proteins

Minerva Biotechnologies Corporation

With SBIR Phase I funding, Minerva Biotechnologies Corp. developed novel nano technologies that enable detailed study of cell surface receptor-ligand interactions that were heretofore not possible.

The technology is based on proprietary nano particles, that we call nano-probes. These are 10 nm gold colloids that we coat with modular self-assembled monolayers (SAMs).

The SAM-coated nano-probes present moieties that bind to commonly used affinity tags, to facilitate the attachment of virtually any biological probe. We chose to modify gold nano particles because they have the intrinsic optical property that when dispersed in a homogeneous solution, the solution appears pink, but if the particles are drawn into close proximity, ie, via interaction of two biological species immobilized on different particles, the solution turns blue. Sensitivity can be increased dramatically by incorporating auxiliary signaling entities (optical or electronic) into the SAM-coating on the nano particles.

The technology is fast and sensitive. The assays are simple, 1-step, no-wash assays, which allow the study of weak interactions. The pre-formed particles are ready to use and customizable. To immobilize a probe peptide, protein, antibody or nucleic acid, one merely adds an aliquot of the probe to an aliquot of the pre-formed particles. Using our electronic detection assays, we were able to study ligand-receptor interactions on the surfaces of live, intact cells. It is critical to study cell surface receptors in their native state. The interactions and behaviors of receptors, which have been taken out of the context of the whole cell, may be irrelevant.

We used our new technologies to attack a very interesting an elusive problem: how does the MUC1 receptor function and how is it linked to tumorigenesis?

Peter E. Barker
National Institute of Standards and Technology
Biomarker Validation Analysis for the EDRN

Peter E. Barker, DeLoise Gambrel-Hocker, Donald H. Atha and Catherine D. O'Connell Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD

Within the NIST DNA Technologies Group, a new program for analytical validation and technology development has been initiated as a component of the NCI's Early Detection Research Network (EDRN). Validation studies focus on nucleic acid analytes in EDRN-supported biomarker assay development for early detection or risk assessment, especially those that parallel ongoing NIST research and technical expertise in DNA diagnostics. Collaborative validation studies in progress include a FISH assay of lung cancer risk that measures molecularly defined, clastogen-induced cytogenetic changes. Prevalidation technology development collaborations between NIST staff and EDRN researchers focus on high throughput screening for serum telomerase activity and high throughput sequence analysis for homoplasmic mutations in mtDNAs for early detection of solid tumors. (Supported by NIST-NCI interagency agreement #Y1-CN-0103-01.)

Robert Basedow
BFGoodrich Corp
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]BFGoodrich Corp (formerly Raytheon), [2]Molecular, Cellular and Developmental Biology Department, Yale

BFGoodrich, in collaboration with Yale University, is developing an imaging spectrometer which will measure, in just a few 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 fields of cancer research and genomics, but will also be capable of being used in a variety of applications, such as the evaluation of assays in high throughput drug discovery experiments. It will be affordable to the average researcher, and 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 BFGoodrich (then Raytheon) for airborne surveillance and reconnaissance applications will be adapted to the laboratory environment. Data analysis techniques, which have been developed for unmixing the spectra of a complex earth scene, will be applied to resolving the signatures in a mixture of fluorophores.

A feasibility and conceptual design study has demonstrated the soundness of the basic approach. A suitable subset of probes has been identified, and key representative characteristics of these have been measured. A radiometric model, tailored to measured probe behavior, has been developed. It has been iterated with the initial instrument concept design to produce an end-to-end model, including spectral unmixing, and an improved hardware design. In the design process, cost has been included as a major constraint. Thanks to recent commercialization of aerospace technologies, almost all components and subassemblies can now be of the commercial-off-the-shelf variety. Hardware trades and selections have been made, and manufacturers published data have been included in the model. The probes themselves can also be made using established techniques, but protocols have been developed to optimize the specific activity of each. Preliminary syntheses of amino-allyl-dUTP labeled DNA has also been carried out, with the objective of increasing the pool of flours suitable for a multi-fluor capability.

Uncertainties of the modeling approach largely involving lack of knowledge of the basic properties of the probes have been estimated. Variability of the kind observed in sets of repeated laboratory measurements on sample probes has also been included, using Monte Carlo methods. The model has yielded false alarm rates as a function of relative concentrations in a 7-fluor mixture, quantification accuracy as a function of one fluor's concentration varying in a mixture of 7 fluors, and system dynamic range (of fluor concentration) as a function of threshold accuracy of quantification. The model has also been used to identify the system's limiting parameters (e.g. detector saturation, signal-to-noise) and refine system design parameters (e.g. laser power, integration time).

Design, fabrication, and in-house testing of the multi-probe instrument are proceeding. The spectral unmixing algorithms which have formed part of the model are being refined

and incorporated into the overall data analysis system. The resulting prototype instrument will be field-evaluated by Yale, and any critical product improvements will be implemented as part of the overall project.

Samuel W. Beenken
University of Alabama

Computer-assisted Cytomorphometric Analysis of Normal Breast Ductal Epithelium, Ductal Carcinoma *in situ* of the Breast (DCIS) and Microinvasive Ductal Carcinoma

Samuel W. Beenken, Neal Poulin, Ph.D, James Crowell, Ph.D, Andra Frost, M.D, William Grizzle, M.., Ph.D, Kirby I Bland, M.D.

University of Alabama

The specific aims of a recent study of 40 archival specimens of ductal carcinoma *in situ* of the breast (DCIS) and/or microinvasive ductal carcinoma included the development of morphometric and architectural indices of cancer and DCIS progression. The analytic strategy for data analysis assumed a continuous model of progression between normal breast tissue, different grades of DCIS and invasive cancer.

5-micron tissue sections were stained using a Feulgen-Thionin stain. Tumor grade was measured according to the Scharf-Bloom-Richardson scale and DCIS was classified according to low, intermediate, and high nuclear grades. Using a high-resolution image cytometer, a digital image of selected nuclei was captured, which contained between 500 and 2,000 individual pixels depending on nuclear size. From the distribution of the individual pixel measurements, over 100 nuclear features were calculated for each nucleus, including DNA content, size, shape, roughness of the nuclear boundary, etc. The nuclei of neoplastic cells as well as the nuclei of surrounding normal-appearing cells were analyzed.

Quality control studies were carried out in order to assess the relative magnitude of errors from various sources, including temperature control of the staining reaction, reproducibility of the staining reaction, effect of section thickness on digital capture, cytometer calibration and stability, and reproducibility. These studies will be presented. Following the quality control studies, discriminant function analysis was used to identify differences in the grade of nuclei from pooled cell measurements. Linear discriminant functions with stepwise variable selection were developed to classify high grade vs. intermediate vs. low-grade tumor nuclei and the discriminant functions so derived were evaluated. These results will also be presented.

Aaron Bensimon
Pasteur Institut, France

Molecular Combing: A technology enabling insight into the dynamic of genomic

instability and DNA replication

Aaron Bensimon[1], John Herrick[1], Ekaterina Svetlova[1], Chiara Conti[1]

Department of Biotechnology, the Biophysics laboratory, Pasteur Institut, Paris, France

The intellectual stance of these studies is the goal of providing a quantitative analysis of events at the level of the single DNA molecule or even the single cell.

The research being pursued 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 technological platform for the genomic study of DNA replication and genetic alterations. This technology involves a method called molecular combing, which is used to straighten and align molecules of genomic DNA on a solid surface.

Molecular combing relies on the action of a receding aie/water interface, or meniscus, to uniformly straighten and align DNA molecules on a solid surface. The ability to comb large molecules at a high density makes long range genomic studies feasible (over 700 kb). The advantages of this approach reside in the reproducibility of the results, their precision (1-4 kb resolution) and the relative ease of analysis afforded by the ability to directly visualize the molecules. Beyond its applications to genomic studies and genetic diseases, it creates new experimental possibilities for research into cancer. We have demonstrated that this is an attractive approach to the study of those phenomena underlying the mechanisms of carciogenesis including microdeletions, inversions and amplifications of specific genetic loci. In a recent study we examined the genomic organization of the proto-oncogene met in renal cell carcinoma. We intend to develop a more general procedure which may contribute for an understanding of the genetic reasons for the tumor development and permit following its evolution in time. Since the density of stretched fibers is high, hundreds of signals are rapidly collected and precisely measured, yielding a statistically reliable analysis. Consequently, the complex organization of a genomic region can be visualized and micro-dissected allowing a molecular genomic classification of a tumor and its evolution in time.

Indeed, as a tool, molecular combing is a versatile approach to a wide range of subjects and question of fundamental interest. This is especially true for the multifaceted domain of DNA replication in higher eucaryotes. Molecular combing, in combination with other higer resolution techniques, permits the identification and mapping of origins of replication on a genome wide basis. Consequently the replication programs of higher eucaryotes can be reliably elucidated by determining the distribution and activities of replication origins over wide regions of the genome. To that effect, we have recently undertaken the mapping and analysis of all origins of replication in the MHC region to verify the validity of this approach for its eventual application in cancer genomic. The longer term goal of our research is to investigate those factors that are implicated in the aberrant replication of the genome in transformed cells. The objective is to elucidate the mechanisms underlying the replication programs in both normal and cancerous cells.

Indeed the replication program differs from one tissue type to another depending on the respective cell?s transcription profile. Hence, our ultimate interest is to establish integrated replication/transcription maps of different tumor type.

The molecular combing method, its biophysical characteristics, and its biological applications to cancer genomic studies will be discussed.

Grant A. Bitter
BitTech, Inc.

Functional Genetic Tests of DNA Mismatch Repair

Grant A. Bitter, Aaron R. Ellison and Joan Lofing

BitTech, Inc.

Genomic instability is associated with cancer progression, and defects in the process of DNA mismatch repair (MMR) is one pathway leading to genomic instability. Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant inherited disease caused by defects in MMR, and mutations in the hMLH1 or hMSH2 genes are responsible for the majority of HNPCC. Defects in MMR have also been observed in a variety of sporadic cancers. In addition to clear loss-of-function mutations conferred by nonsense or frameshift alterations in the coding sequence or by splice variants, genetic screening has revealed a large number of missense codons (25% of all alterations observed) with less obvious functional consequences. The ability to discriminate between a loss-of-function mutation and a silent polymorphism is important for genetic testing for inherited diseases like HNPCC where there exists opportunity for early diagnosis and preventive intervention. In this project, quantitative in vivo DNA mismatch repair (MMR) assays in the yeast Saccharomyces cerevisiae are utilized to determine the functional significance of amino acid replacements observed in the human population.

Missense codons previously observed in human genes were introduced at the homologous residue in the yeast MLH1 or MSH2 genes. This study also demonstrated feasibility of constructing genes that encode functional hybrid human-yeast MLH1 proteins in which regions of the yeast protein were replaced with the homologous region from the human protein. The genes encoding functional hybrid MMR proteins allow determination of the in vivo effects of codon changes at residues that are not conserved in the yeast gene. Three classes of missense codons were found: 1) complete loss-of-function, i.e. mutations; 2) variants indistinguishable from wild-type protein, i.e. silent polymorphisms; and 3) functional variants which support MMR at reduced efficiency i.e. efficiency polymorphisms. There was a good correlation between the functional results in yeast and available human clinical data regarding penetrance of the missense codon in HNPCC. The results of this study raise the intriguing possibility that differences in the efficiency of DNA mismatch repair exist between individuals in the human population due to common polymorphisms. Future work will investigate this possibility by further

mutation analysis. If individual differences in the efficiency of MMR exist due to common polymorphisms, it would predict differential sensitivities to cancer development. Elucidation of such relationships will facilitate implementation of cancer prevention strategies.

Kirby I. Bland
University of Alabama

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Samuel W. Beenken, M.D., Neal Poulin, Ph.D., James Crowell, Ph.D., Andra Frost, M.D., William Grizzle, M.D., Ph.D., Kirby I. Bland, M.D.

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Steven A. Bogen
CytoLogix Corporation

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

Steven A. Bogen and Greg Testa

CytoLogix Corporation, Cambridge, MA

Modern methods for the genetic analysis of cancer include hybridization assays to tissue sections, cells, and arrays. Each of these techniques has in common the fact that they are performed on an optically clear flat surface, such as a microscope slide. These assays require that a small amount of reagent is spread over a planar surface and heated while preventing evaporation. Traditionally, this has often accomplished by sealing a coverslip over the sample with rubber cement or nail polish. However, that process is not readily amenable to automation. Although the problem seems relatively straightforward, it has been particularly difficult to solve because the assay procedure entails a high surface to volume ratio. Both friction and evaporation occur at liquid-surface interfaces, making it difficult to obtain even reagent spreading with low reagent volumes, without evaporation in a fashion that can be performed robotically. Under Phase I SBIR funding, CytoLogix demonstrated feasibility of a microfluidics technology that addresses these technical constraints. This microfluidics technology involves a means for fluid exchange in a capillary-thick chamber. The main technical barrier has been filling the microchamber with reagent without entrapping air bubbles over the sample. CytoLogix plans to further develop this technology into a walk-away, completely automated instrument.

Gerry Boss
University of California, San Diegos

Automated Measurement of Ras and Rho Activation in Cancer

Gerry Boss[1], Anna Dreilinger[2], David Gough[3], James Harrell[1], Stephen Jones[4], Stephen Qualman[5], Anne Wallace[6], and Linda Wasserman[2]

[1]Departments of Medicine, [2]Pathology, [3]Bioengineering, and [6]Surgery, and [4]Electronics Shop, University of California, San Diego, La Jolla, CA, and [5]Department of Pathology, Ohio State University, Columbus, OH

Ras and Rho transmit pro-proliferative and cellular transforming signals when appropriate ligands bind to growth factor receptors on the plasma membrane; both proteins cycle between an active GTP-bound state and an inactive GDP-bound state. We devised a method to measure Ras activation (the ratio of Ras-bound GTP over Ras-bound GTP plus GDP) in human tumors and found that Ras is highly activated in a significant number of neuronal tumors and in breast, lung, and ovarian cancers, even in the absence of a genetic mutation in the ras gene. Assessing Ras activation provides information not only about the basic biology of a tumor, but it may also have therapeutic importance because a large number of Ras inhibitors are under development or are already in clinical trials; in cell culture and animal models, these drugs are most

effective when Ras is in an activated state. Some of the agents designed to disrupt Ras function have been found to exert their inhibitory effects on cell growth by inhibiting Rho and Rho may be involved in the development of metastases. It is likely, therefore, to be of clinical value to assess the activation states of Ras and Rho as part of developing specific chemotherapeutic regimens based on the molecular alterations found in a tumor.

Graham J.R. Brock
University of Glasgow, UK

Identification of m5CpG alterations associated with breast carcinomas

Graham J.R.

Brock Institute of Biomedical and Life Sciences; Dept. Molecular Genetics, University of Glasgow, UK

Objectives
A: To fractionate DNA fragments retaining those with the large numbers and densities of m5CpGs (~5% of the genome). B: To establish the efficiency of a subtractive hybridization procedure. C: To isolate the densely methylated fraction from matched pairs of normal and tumor tissue then use the subtractive hybridization procedure to isolate those sequences with altered methylation patterns.

During tumourigenesis, normal genomic methylation patterns are altered by the addition of a methyl group to the 5 cytosine of CpG dinucleotides. Well-characterized examples include the hypermethylation of the ordinarily unmethylated GC-rich regions known as CpG islands (CGIs). Whether methylation changes occur as a cause or effect is currently unclear since the earliest changes have yet to be defined. However, once established, aberrant methylation changes are faithfully reproduced in all progeny cells, making such alterations potential biomarkers for tumour identification and classification.

To examine those GC-rich regions, altered during tumourigenesis, a novel method was developed which utilized a methyl CpG-binding domain (MBD) column. The MBD column has a strong affinity for densely methylated sequences, allowing these to be purified from bulk genomic DNA. However, in addition to methylated CGIs large numbers of repetitive sequences (e.g. LINEs and SINES) are both GC-rich and methylated and therefore bind to the MBD column. To remove both high and low copy number repeats a process of subtractive hybridization followed by linker attachment and amplification was used. Following optimisation of the method through recovery of a plasmid spike, a library of sequences (whose methylation status is altered in DNA extracted from a poorly differentiated tumour and its adjacent normal tissue counterpart) was then generated. With the DNA derived from the tumor being used as tester and DNA derived from normal tissue used as driver. Of 61 clones fully sequenced to date, 31 have the GC content and CpGobs/exp of a CGI. Database searches with these sequences indicate no homology to known CGIs, however, analysis of regions from

which the clones are derived (using the GRAIL/cpg program) demonstrates that many of these sequences are fragments of predicted CGI's. Analysis of the methylation status of these regions in matched pairs of tumour and adjacent normal samples is currently underway.

These results and future potential uses of libraries derived from matched pairs of normal and tumour DNA will be discussed. The libraries will be used to generate genomic arrays of sequence whose methylation status is altered during tumourigenesis. These arrays will then be utilized in the genome wide analysis of alterations in methylation patterns in different grades of tumours and to identify specific patterns of methylation and any association with clinically defined tumour stage and grade. Finally the method will be employed to investigate loss of methylation by using the DNA derived from a tumor as the driver at the subtractive hybridisation stage.

Marcel Bruchez
Quantum Dot Corporation
Development of Quantum Dot Based Probes for Multiplexed

R. Hugh Daniels, Marcel Bruchez, Xingyong Wu, Kari Haley, Christopher Ng, Yanzheng Xu, and Huayong Yong

Quantum Dot Corporation

Semiconductor quantum dots are a new class of fluorescent probe that can be used for simple multiplexing with a single excitation source. These materials have been developed for detection of progesterone and estrogen receptors, P53, cytokeratin 8/18 and Her-2 in sub-nanogram quantities, and have been used to specifically stain these markers in both human breast cancer cell lines and tissue sections. These probes have been used in model assays to demonstrate the ability and simplicity of multiplexing, and multiplexed analysis of breast cancer tissues are currently underway. The probes developed show substantially improved photostability in the cellular milleu, substantially higher sensitivity and are significantly more stable to archiving than similarly stained materials using fluorescent dyes. These quantum dot probes will be developed into a more complete platform for cancer sub-classification and diagnosis.

Tauseef R. Butt
LifeSensors Inc.
Biosensors: Molecular Profiling of Cancer by Encapsulating Ligand Mediated Human Nuclear Receptor Function in Cell-Based Sensors

Stephen Weeks, Hiep L. Tran, and Tauseef R. Butt

Research and Development, LifeSensors Inc, Malvern, PA

Application of human nuclear receptor as sensors in molecular profiling individuals with high risk for progression of specific type of cancer is very promising. In addition, quantifying response to therapy with Biosensors also holds a great promise. Ligand dependent function of many human nuclear receptors has 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 panel of yeast cells engineered with several human nuclear receptors (Biosensors) are ultra-sensitive to ligands and hormones. Receptor regulating activities from human sera of control and cancer patients can be. We have? monitored using the panel of functional microarrays called LifeSensors used human estrogen receptor as a model system to develop an ultra-sensitive estradiol and phytoestrogens?estrogen sensor. It has been shown that 17 transactivation rank order of potency was remarkably similar in yeast and human HepG2 cells. Yeast cells demonstrated an order of a magnitude higher efficacy for variety of compounds as compared to human cells. Application of this technology to monitor pre-disposition to disease and therametrics will be discussed.

Susan Castillo
SRA International
An XML Extension to MultiCluster

Ali Al-Timimi, Eric Cahoon, Susan Castillo, Venkat Chalasani, and Scott Bennett

SRA International, Fairfax, VA

The amount of data generated by microarray gene expression experiments can be enormous. To better understand this data, computationally intensive analysis tools are needed. MultiCluster brings together multiple methods for analysis of microarray data.

As it becomes more and more imperative for researchers to share these data cooperatively, the need for a standard method for data exchange becomes paramount. Recently, a number of XML standards for the exchange of gene expression data along with the associated gene and experiment annotation have been proposed. Among these standards is MAML (Microarray Markup Language) (http://www.mged.org).

We have added to MultiCluster a Java transformation tool that maps MultiCluster data sources to the MAML DTD and subsequently converts the data to XML. With this tool, MultiCluster users can rapidly create MAML XML documents through a user-friendly interface.

Jeffrey J. Chalmers
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

Magnetically based cell separation technologies have become commonly used techniques to enrich and/or separate cells of interest from a heterogeneous 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. With respect to the first aspect, we have conducted studies which characterizes the degree to which several human cancer cell lines, and tissue from a primary tumor, can be labeled with both fluorescently labeled, and magnetically labeled antibodies. We used both FACS and a new type of instrument that we have developed, Cell Tracking Velocimetry, CTV, to conduct these studies.

With respect to the second and third aspect, we have developed two flow-through magnetic cell separators. One separator, the Quadrupole Magnetic cell separator, QMS, can separate immunomagnetically labeled cells at a feed rate of 107 cells/s. The second separator, the Dipole Magnetic Flow Sorter can "Fractionate" cells in to different sub-populations based on the cells magnetophoretic mobility (degree of immunomagnetic labeling). Various cell mixtures are being separated with these two systems as well as commercial, batch systems. Measures of the performance of these systems are being developed using a variety of analytical methods including FACS and CTV.

Wing C. (John) Chan
University of Nebraska Medical Center

Molecular Analysis of a Large Series of B-Cell non-Hodgkin's Lymphoma

Wing C. (John) Chan

University of Nebraska Medical Center

Tumors derived from the same cell type and having similar morphology may nevertheless have a distinctly different clinical behavior and response to therapy. Differences in the genetic lesions in these tumors, as reflected by their gene expression profiles, will provide insight into the mechanisms underlying the divergent clinical

spectrum that is observed. Comparative genomic hybridization (CGH) and spectral karyotyping (SKY) and highly complementary novel techniques that examine the entire genome for genetic abnormalities and can supplement and extent conventional cytogenetic studies. In addition, the recently developed high density cDNA microarray technology is a very promising method for displaying the pattern of gene expression in tumor tissues. These powerful technologies with their associated informatic systems are now available for translational research. In order to evaluate the information generated by these technologies, an adequate number of well-characterized tumors with detailed clinical data must be available. We propose a multi-institutional, comprehensive molecular analysis of a large series of B-cell non-Hodgkin's lymphoma (NHL).

Britton Chance
University of Pennsylvania
Molecular Beacons

Britton Chance

University of Pennsylvania

The remarkable scope of the IMAT meeting, together with the NCI commitment to the program, the perspectives of Dr. Klausner, together with the teachings on RNA, protein expression, protein structure and function make this a must for anyone attempting to do today's biochemistry of cancer.

The fact that molecular beacons are now planned to recognize specific genetic expressions of cancer makes this program of special interest to those working on the UIP. It is hoped that not only the PI, but also those designing contrast agents would be able to attend this fascinating session.

Mark R. Chance
Albert Einstein College of Medicine
Novel Proteomics Methodologies for Examining Protein-Protein Interactions in

Michael Brenowitz and Steve Almo

Center for Synchrotron Biosciences, An NIH Resource Center and Albert Einstein College of Medicine, Bronx NY 10461

Radiolysis of water by synchrotron X-rays generates oxygen-containing radicals that undergo reactions with solvent accessible sites of macromolecules inducing stable covalent modifications or cleavage on millisecond timescales. The extent and site of these reactions are determined by gel electrophoresis for nucleic acids and mass spectrometry analysis in the case of proteins. The Albert Einstein Center for Synchrotron Biosciences (www.aecom.yu.edu/home/csb/) has been developing

synchrotron footprinting technology for the last several years with the aim of determining detailed structure and dynamics information in solution for large macromolecules and their complexes (Sclavi et al., Science, 279: 1940-1943 1998, Maleknia, et al. Anal. Biochem. 289: 103-115, 2001).

The footprinting data is used to construct a quantitative map of solvent accessibility at individual reactive sites. The experiments can be performed in both in an equilibrium configuration with a matrix of proteins to examine protein-protein interactions in a high-throughput fashion and in a time-resolved manner to provide kinetic rate constants for formation of protections for individual sites within a macromolecule. The application of this synchrotron footprinting technique to the study of DNA-protein complexes and interactions of actin binding proteins will be presented. These model systems will drive the technology to provide general methods relevant to studying protein-protein interactions in cancer biology. Specifically, we will examine a library of C. elegans proteins implicated in DNA damage and cell-cycle checkpoint that have been selected by high-throughput screening and expressed and purified by high-throughput proteomics methods.

Zunxue Chang
University of Minnesota

Cloning and Characterization of the Biosynthetic Gene Clusters for Curacin A and Barbamide from Cyanobacterium Lyngbia majuscula

Zunxue Chang[1], Patricia Flatt[2], William H. Gerwick[2], and David Sherman[1]

[1]Department of Microbiology, University of Minnesota, Minneapolis, MN, [2]College of Pharmacy, Oregon State University, Corvallis, Oregon

Curacin A was isolated as a major lipid component of a marine cyanobacterium strain Lyngbya majuscula for its high brine shrimp toxic and anti-proliferative activities. Its ability to inhibit cell growth and mitosis and to bind rapidly and irreversibly to the colchicine site of tubulin make it a potential anticancer drug lead. A chlorinated lipopeptide, barbamide, was also extracted from the same strain for its high ichthyotoxicity. Screening of the genomic library of L. majuscula constructed in cosmid vector pOJ446 using the PCR-amplified polyketide synthase (PKS) fragments as a probe identified sixty positive clones. Two groups (A and B) were assigned to be responsible for the biosynthesis of curacin A and barbamide, respectively by three rounds of Southern hybridization using the PCR-amplified PKS and non-ribosomal peptide synthetase (NRPS) fragments as probes, and their DNA sequence was determined. Analysis of the 80-kb assembled DNA sequence of three cosmids (group A) revealed nine large ORFs responsible for ten modules of PKS and NRPS, and three small ORFs for HMG-CoA formation, which is presumed to be an intermediate in curacin biosynthesis. Domain organization of the PKS and NRPS modules is consistent with the chemical structure of curacin A. Analysis of the DNA sequence of another cosmid (group B) revealed three modules of NRPS and one module of PKS. Combined

with the domain organization, this gene cluster is believed to be the barbamide gene cluster. The several small ORFs upstream of the first NRPS module is presumed to specify chlorination and modification of leucine, the initiation unit of barbamide biosynthesis. The heterologous expression of curacin A, the adenylation domains and the whole gene cluster of barbamide in E. coli and Streptomyces venezuelae are in progress.

Mark Chee
Illumina, Inc.

Randomly Self-Assembled Bead-Based Arrays for Gene Expression Analysis

Mark Chee, Tim McDaniel, Shawn Baker, Semyon Kruglyak, Francisco Garcia, Kenneth Kuhn, Csilla Fenczik, Kevin Gunderson, and Jian-Bing Fan

Illumina, Inc., San Diego, CA

Self-assembled arrays of bead-based sensors have been developed. Each bead contains oligonucleotide probes that can hybridize with high specificity to complementary sequences in a complex nucleic acid mixture. The identity of each bead in the random array is determined by a hybridization-based decoding procedure. By formatting the miniaturized arrays into a matrix that matches a 96-well microtiter plate, many samples can be processed efficiently in parallel. We are using this platform to develop assays in the areas of SNP genotyping, gene expression profiling, and protein analysis. Progress in the area of gene expression profiling will be discussed.

Gary A. Churchill
The Jackson Laboratory

Statistical Design and Analysis for Gene Expression Microarrays

Gary A. Churchil

The Jackson Laboratory

Gene expression microarrays are an innovative technology with enormous promise to reveal the function of genes in normal and diseased tissues. Although the potential of this technology has been clearly demonstrated, many important and interesting statistical questions persist.

The problem of making relative comparisons among large numbers of samples using heterogeneous experimental conditions is not new. Indeed there is a history of 100 years of research in agricultural experimentation which is relevant and can be applied to the problem of designing microarray experiments.

We advocate greater attention to experimental design issues and a more prominent role

for the ideas of statistical inference in microarray studies.

It is our premise that good bioinformatics begins before one goes to the bench. Experiments that are well designed can be used to answer not only the questions which they were specifically designed to address they can also be "mined" for useful information which was not anticipated by in the original experiment. Sound statistical design is crucial to ensure that these goals can be achieved. We will illustrate these points by contrasting the analysis of microarray data obtained on the same set of samples using both standard and novel experimental designs.

John Condeelis
Aecom

Novel imaging methods for gene discovery in cancer

J. Condeelis

Aecom

We have developed metastasis models in rats and mice that permit real time multiphoton-based imaging of the behavior and interactions of metastatic tumor cells in the primary tumor in vivo. Certain mouse models are clinically relevant in that they resemble human breast tumors in etiology and histology. Intravital imaging results indicate that tumor cell chemotaxis leads to the accumulation of tumor cells around, and their polarization toward, blood vessels in primary breast tumors and is correlated with efficient intravasation (Cancer Research 60:2504-2511, 2000). Viable tumor cells can be collected from the blood of rats with metastatic tumors with 90-fold greater frequency than from rats with non-metastatic tumors. Intravital imaging of GFP labeled tumor cells was used to identify blood burden in the vessels at the edge of the tumor. Six-fold more tumor cells are observed in vessels per minute per 250 um square field of metastatic tumor compared to matched preparations of non-metastatic tumors. Since tumor cell polarization toward blood vessels and chemotaxis are not observed in matched non-metastatic tumors, polarization and chemotaxis of tumor cells toward blood vessels are proposed to be important in invasion and metastasis. To extend these observations to a mechanistic level, we have used chemotaxis of tumor cells in vivo to advantage to collect subpopulations of motile and chemotactic tumor cells from primary breast tumors. Needles containing chemoattractants and matrigel are used to collect motile tumor cells in vivo as a pure population suitable for further analysis.

Christopher H. Contag
Stanford University

Visible Animal Models of Neoplastic Disease

Christopher H. Contag[1], Leonard A. Herzenberg[2], Robert S. Negrin[3], Leonore A. Herzenberg[2]

[1]Department of Pediatrics and Microbiology and Immunology, [2]Department of Genetics, and [3]Department of Medicine, Stanford University, Stanford CA

Imaging reporter gene expression in living animals provides critical spatiotemporal information about changes in cell growth, cell trafficking and gene expression during normal and disease processes. We have added another dimension to this powerful in vivo assay by coupling it with equally powerful fluorescence methods for ex vivo identification of cells in suspension or tissue sections, through the use of multifunctional reporter genes. Together, these technologies enable effective methods for evaluating immune surveillance of neoplastic disease, and improving cell-based and other anti-cancer therapies. We have described in vivo tumor-host immune interactions based on real time observations of trafficking and proliferation of immune and tumor cells in intact animals. We have designed a build a series of multifunctional reporter genes that can be used for in vivo trafficking studies and in ex vivo assays (fusion proteins comprised of luciferases and fluorescent proteins) that are detectable in vivo by bioluminescence and ex vivo by fluorescence.

Jose Costa
Yale University School of Medicine
Technologies for Mutational Load Distribution Analysis

Daniela Ladner[1], David Ward[2], Paul Lizardi1, and Jose Costa[1]

[1]Department of Pathology, Yale University School of Medicine, [2]Department of Genetics, Yale University School of Medicine, New Haven, Connecticut

There is an urgent need for biomarkers capable of identifying patients at risk during early phases neoplasia in pancreatic, breast, and colon tumors. Having access to surrogate samples for the analysis of these tissues provides an opportunity for the development of noel biomarkers whose status can be assessed through non-invasive to minimally invasive procedures, using currently available technologies. We propose that quantitating the proposition of mutated cancer alleles (cells) in a population of somatic cells, and measuring (with sufficient statistical power) the degree of diversity at specific gene loci, will accurately reflect the risk of cancer and is likely to emerge as a biomarker that can be validated prospectively and applied widely. We refer to this analysis as Mutational Load Distribution Analysis (MLDA). Surrogate tissue samples containing a sufficiently small number of cells, will enable us to perform MLDA analysis during the preneoplastic stages of tumor development. We will use technologies that lend themselves well to quantitative analysis.

Sandra L. Dabora
Brigham and Women's Hospital
Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2 compared with TSC1 disease in multiple organs

Sandra L. Dabora[1], Sergiusz Jozwiak[2], David Neal Franz[3], Penelope S. Roberts[1], Andres Nieto[1], Joon Chung[1]), Yew-Sing Choy1,[4], Mary Pat Reeve[1], Elizabeth Thiele[4], John C. Egelhoff[3], Jolanta Kasprzyk-Obara[x2], Dorota Domanska-Pakiela[2], and David J. Kwiatkowski[1]

[1]Brigham and Women's Hospital, Boston, MA, [2]Children's Memorial Hospital, Warsaw, Poland, [3]Children's Hospital Medical Center, Cincinnati, OH, and [4]Children's Hospital, Boston, MA

Tuberous sclerosis (TSC) is a multisystem hamartoma syndrome, caused by mutations in either of two tumor suppressor genes, TSC1 and TSC2. We have performed comprehensive mutation analysis in 224 index TSC patients and correlated mutation findings with clinical features. DHPLC, long-range PCR and quantitative PCR were used for mutation detection. Mutations were identified in 186/224 (83%) of cases, comprising 138 small TSC2 mutations, 20 large TSC2 mutations, and 28 small TSC1 mutations. A standardized clinical assessment instrument covering 16 TSC manifestations was used. Sporadic patients with TSC1 mutations had on average milder disease compared with patients with TSC2 mutations despite being of similar age. They had a lower frequency of seizures and moderate-severe mental retardation, fewer subependymal nodules and cortical tubers, less severe kidney involvement, no retinal hamartomas, and less severe facial angiofibroma. Although there was overlap in the spectrum of many clinical features of patients with TSC1 versus TSC2 mutations, some features (grade 2-4 kidney cysts or angiomyolipomas, forhead plaques, retinal hamartomas, and liver angiomyolipomas) were very rare or not seen at all in TSC1 patients. Thus both germline and somatic mutations appear less common in TSC1 than in TSC2.

Norman J. Dovichi
University of Washington

The Single Cell Proteome Project: Cancer Prognosis

Norman J. Dovichi, Shen Hu, Amy Dambrowitz, Le Zhang, David Michels

Department of Chemistry, University of Washington, Seattle, WA

The objectives of this proposal are to generate two-dimensional protein map from single cancer cell, to correlate the map with the phase of the cell in the cell cycle, and to multiplex the instrument to analyze several cells in parallel.

Our methods focus on the development of capillary electrophoresis instrumentation with ultrasensitive laser-induced fluorescence detection.

We have achieved several results over the past two years. We have generated the first protein map of a single cell. This map was based on 1-dimensional free solution electrophoresis of an HT29 adenocarcinoma cell. Since that work, we have also generated single cell protein maps based on 1-dimensional free solution electrophoresis

of a single-cell C. elegans embryo and single E. coli cells. In addition to the free solution electrophoresis experiments, we have generated single cell proteome maps based on 1-dimensional capillary gel electrophoresis of HT29 cells. We have modified our instrument to determine the phase of the cell in the cell cycle before analysis, and we have correlated cell cycle with capillary gel electrophoresis protein maps. Last, we have developed a prototype 2-dimensional capillary electrophoresis instrument for fully automated protein analysis.

Our plans for the last year of this grant are to incorporate our two-dimensional electrophoresis technology into the single cell analytical instrument. We will also develop a multiple capillary system to analyze several cells simultaneously.

Daniel L. Farkas
Univ. of Pittsburgh and Carnegie Mellon Univ.

Cancer Molecular Analysis by AOTF Multispectral Imaging

Daniel L. Farkas [1,2], Elliot Wachman [2], and Stanley Shackney[3]

[1]Univ. of Pittsburgh, [2]Carnegie Mellon University , and [3]Allegheny General Hospital, Pittsburgh PA Department of Biotechnology, the Biophysics laboratory, and Pasteur Institut, Paris, France

Objective: Develop the capability to study the accumulation of multiple abnormalities per cell at the molecular level, for improved cancer diagnosis and individualized treatment.

Methods: By focusing our proprietary technologies towards the program's goals we will (a) build the next-generation imaging cytometry instrument, able to bypass previous limitations; (b) concentrate on the imaging of 5-10 molecular species simultaneously within the same cancer cell, with a new multispectral imaging instrument incorporating our acousto-optic tunable filters (AOTFs) and their unique simultaneous spatio-temporal and spectral capabilities. Geared towards the imaging of human solid tumor-derived cells, with sub-cellular resolution, this will be a highly flexible, computer-controlled instrument combining the best features of a research microscope, a spectrometer and an imaging cytometer. The innovation consists in bringing versatile tunability to multi-parameter imaging cytometry, to increase the number of entities resolved per cell and their quantitation far beyond what is possible today, for breast and lung cancer research. The research being pursued 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 technological platform for the genomic study of DNA replication and genetic alterations. This technology involves a method called molecular combing, which is used to straighten and align molecules of genomic DNA on a solid surface.

Results: We concentrate on the improvement of digitally controlled spectral devices using AOTF technology, and integrate them on both the excitation and emission sides

of a research microscope. The emphasis is on fluorescence, due to its very high specificity for labeling intracellular features, and we aim to reproducibly resolve probes with highly similar but non-identical spectral signatures. Moreover, background subtraction will be based on spectral features, yielding a more elegant way of disposing of unwanted contributions arising from autofluorescence or the presence of unconjugated dye. The system compares very well in performance with all alternative technologies, and the difference between multi-wavelength and spectral imaging goes beyond that pertaining to the number of image acquisition wavelengths utilized. Spectral imaging is differentiating between various contributions by their global spectral signature, thus being a more quantitative, and sophisticated method. Multispectral imaging is, in our definition, spectral imaging with added versatility: both excitation and emission wavelengths could be spectrally selected, scanning could be done with either or both, including a tandem scanning, where excitation and emission are varied simultaneously, with a fixed, but selectable wavelength gap between them. We will demonstrate the unique discrimination thus achieved by imaging four very closely spaced dyes under the same filter set. Additionally, in multispectral imaging one can vary critical experimental parameters such as exposure times at various wavelengths, very helpful when trying to equalize signal-to-noise in datasets by longer exposure at less sensitive wavelengths.

Plans: We aim to (1) build a prototype instrument with the desired new functionality; (2) implement a second, more user-friendly workstation in our cancer research laboratories; (3) test the latter instrument in experiments focusing on molecular-level prognostic factors for tumor progression within single cells of individuals' tumors; (4) elucidate critical sequences of genetic evolutionary changes in solid tumors that are responsible for increasing cancer aggressiveness; (5) identify the steps in the sequence that are most closely associated with cellular acquisition of the capacity to metastasize; (6) develop a productized, customizable version of the instrument embodying the new technology, multispectral imaging, ready for use on an array of problems by other researchers; and (7) develop a practical overall approach for the timely performance of relevant measurements on individual tumors, analysis of the data, and characterization of each tumor with respect to the degree of its advancement along its particular genetic evolutionary pathway, so that this information can be used for prognosis and adjuvant treatment planning. The ultimate clinical challenge is the elimination of false negatives and false positives in diagnostics, leading to individually optimized treatment and very significant savings. Overall, our focus, innovation and expected competitive advantage will consist in bringing together the specificity of fluorescence with the relevance and diagnostic importance of measuring multiple labels in the same cell, and with the ability to do so for 5-10 fluorophores, using the new technology of AOTF multispectral imaging.

David A. Fishman
Northwestern University
EDRN National Ovarian Cancer Early Detection Program

David A. Fishman

Northwestern University

Epithelial ovarian cancer (EOC) is the fourth leading cause of death in American women due to our present ability to detect only late (III/IV) stage disease. Currently 75% of all EOC patients are diagnosed with late stage disease with only a 12-15% 5-year survival despite aggressive cytoreductive surgery and chemotherapy. However, 90% of women diagnosed with early disease (Stage I) are alive 5 years after less morbid surgery and chemotherapy. The National Ovarian Cancer Early Detection Program was specifically established to clinically apply our understanding of the biochemical, genetic and molecular basis of ovarian carcinogenesis, invasion and metastasis to address the problem of early detection of epithelial ovarian cancer. This research consortium includes over 100 clinicians and scientists worldwide. The enhanced understanding of ovarian cancer biology has led to the identification and detection of specific genetic, molecular and serum biomarkers in women with ovarian cancer that may have clinical utility in the evaluation of women deemed at increased risk for the development of this disease. Increased risk is assigned to those women with either a personal history of breast cancer (4X increase), a family history of affected first-degree relatives (2-7x increase), membership within a recognized inherited malignancy syndrome (40-60% increase), or the presence of an inherited BRCA mutation (16-100%). The newly developed Ovarian Pap test provides cytological samples for pathological examination as well as molecular, genetic, and biochemical analysis.

The biochemical and molecular analysis of tissue and tumor samples for changes in patterns of gene expression of a select subgroup of growth regulatory molecules that already have been implicated in EOC growth and tumor progression is under investigation. One experimental approach used is the high throughput molecular genetic analysis of tissue and tumor samples using state-of-the-art methodologies. As our studies progress, we intend to correlate the results of these high throughput studies with our expression and functional analyses, thereby maximizing the clinical application of this new information. The metastatic process of cellular adhesion, migration, extracellular matrix degradation, invasion into host parenchyma, proliferation, and neovascularization are influenced by numerous regulatory molecules, such as epidermal growth factor (EGF) and receptors (EGF-R/ErbB isoforms such as p110), urinary-type plasminogen activator (uPA) and receptor (uPAR), matrix metalloproteinases (MMP), and lysophospholipids (such as LPA, LPC). Levels of lysophosphatidic acid (LPA) are elevated in the plasma of patients with ovarian carcinoma including 90% of patients with stage I disease, suggesting that LPA may promote early events in ovarian carcinoma dissemination. Activation and cell surface expression (yet not gene, RNA, or protein expression) of MMPs are also upregulated in malignant ovarian epithelium, and we have reported a direct role of MMPs in intraperitoneal invasion and metastasis. Using three-dimensional type I collagen cultures or immobilized a1 integrin subunit-specific antibodies, we previously demonstrated that a1 integrin clustering promotes activation of proMMP-2 and processing of membrane type 1 (MT1-)-MMP in ovarian cancer cells. LPA increased cellular membrane fluidity and adhesion to type I collagen and a1 integrin expression with significant upregulation of MMP-dependent proMMP-2 activation, leading to

enhanced pericellular MMP activity.

As a result of increased MMP activity, haptotactic and chemotactic motility, *in vitro* wound closure, and invasion of a synthetic basement membrane were enhanced. These data indicate that LPA contributes to metastatic dissemination of ovarian cancer cells via upregulation of MMP activity and subsequent downstream changes in MMP-dependent migratory and invasive behavior. It is our hope that the ability to simultaneously evaluate a single patient for multiple biomarkers, all of which have been demonstrated to be significant in ovarian carcinogenesis and metastases, should translate into a means for identifying early stage disease.

Paolo Fortina
Children's Hospital of Philadelphia and University of Pennsylvania School of Medicine

Four Color Array-bound SNP/mutation Detection in Cancer

Paolo Fortina[1], Taku Sakazume[1], David Graves[2], Marty Johnson[3], Penny Dong[3], Kathleen Delgrosso[4], and Saul Surrey[4]

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Development of cost-effective, high-throughput genotyping methods will facilitate molecular analysis of normal and diseased states. This initiative focuses on detection of SNPs and point mutations in cancer. Milestones for phase 1 are as follows: 1) Development of cost effective 4-color array bound single nucleotide primer extension (SNE) with signal enhancement, including algorithms for deconvolution of spectral overlap; 2) Scale-up for simultaneous detection of 30-50 interrogated sites and technology validation compared to other methods; and, 3) Use of dendritic surfaces or other methods to reduce costs.

We recently reported a 2-color array-based SNE assay for mutation detection (Eur. J. Hum Genet. 8: 884-894, 2000), and are working on a 4-color assay which will require spectral deconvolution due to dye emission overlaps. A deconvolution algorithm was developed by first ascertaining whether dyes in solution spectrally behaved like array-bound oligo-extended dyes after SNE. Spectral properties were compared after excitation at 488 nm for each of the 4 separate Big Dyes (ABI/PE) in solution, as well as following single color array-based SNE. We found similar normalized spectral emissions comparing the 2 different approaches, but only when the array was scanned in a hydrated state. We next investigated whether there was any quenching when the 6 possible 2-color dye combinations were mixed. We tested this by doing all 6 possible 2-color array-bound extensions so that both dyes were incorporated at the same register.

Our results showed minimal quenching; and, therefore deconvolution software was developed, and is being used for assessing spectral overlap for multicolor SNE on arrays.

We next investigated use of polyamidoamine starburst dendrimers as spacer molecules that can be attached to glass, to increase surface density of oligonucleotides on arrays and to enhance SNE signals. Surface coverage was varied using different generations of dendrimers whose size and number of reactive groups were directly related. Adsorption of PCR products indicated optimal dendrimer concentration versus generation utilizing this surface chemistry. We are now applying use of these surfaces in array-bound multicolor SNE reactions, and are testing whether spacers and amino-modification can be eliminated when using various slide surfaces in an effort to reduce assay cost.

We are also testing a new ASO DNA chip which allows melting temperatures at each array register to be independently controlled. Preliminary results with a prototype thermal gradient chip indicate optimum hybridization conditions can be defined for each probe/target reaction on the array.

Aims for phase 2 include: 1) Application of SNE compared to other genotyping strategies to type SNPs within and flanking the minimal deleted region in neuroblastoma patients; and, 2) Identification of DNA changes in the mutation cluster region of the APC gene in patients with colorectal cancer.

Development and validation of array-bound approaches for monitoring DNA changes in cancer should facilitate high throughput, parallel processing of samples for critical human cancer genes. In addition, it will help to rapidly define the molecular basis of specific malignancies and eventually provide a foundation for rational molecular assessment of therapeutic approaches for treating these diseases.

Jacques R. Fresco
Princeton University

Fluorescent TISH Probes for Cancer-relevant Sequences

Jacques R. Fresco[1], Nina Dolinnaya[1], Olga Amosova[1], Alex Weis[2], and Tamas Bakos[2]

[1]Department of Molecular Biology, Princeton University, Princeton, N.J., [2]Lipitek International, San Antonio, TX

Gene amplification, substitution and deletion mutations are associated with the molecular pathology of various malignancies, including breast, cervical and gastric cancers, as well as colon and lung cancers and tumors of the nervous systems. Multidrug resistance of cancer cells is also associated with gene amplification. Amplification of erbB-2 (HER-2/neu) and N-myc genes is particularly correlated with poor prognosis in breast and cervical cancers and neuroblastoma, respectively.

Methods that could simply and reliably detect such aberrations and quantitate them can therefore be of great value for diagnosis, for following the efficacy of treatment, and for reliable prognosis. Currently, detection of point-mutational events represents a major experimental effort. In the case of gene amplification as well, the methods are arduous, require relatively large samples, and they are of variable reliability. This project has a two-fold aim. One is to apply the methodology of TISH (third strand *in situ* hybridization of fluorescent probes via triplex formation, which avoids the need for DNA denaturation) for the cytogenetic quantitative analysis of these aberrations. TISH has three major advantages: greater sensitivity and quantitative reliability, and importantly, greater sequence specificity. The second parallel aim is to develop suitably intense and non-quenching fluorescent TISH probes. For this purpose, a major effort will be mounted to develop dendritic nuclei with fluors attached by rigid linkers that prohibit their interaction, therefore preventing the quenching of fluorescence. In this way, it is hoped to enhance the sensitivity of fluorescent probe detection by 1-2 orders of magnitude, thereby assuring that TISH probes can be reliably used for analyzing amplified genes and ultimately for detection of mutations in single-copy genes *in situ*.

In the first year of this investigation, one aim is to develop probes for the HER-2/neu gene. Nine different deoxynucleotide probes have been designed for the homopyrimidine targets present in the gene. Three of these have?9 homopurine already been synthesized and tested.

Simultaneously, an amplified fluor with three fluorescent moieties linked to a rigid adamantane core has been prepared, and efforts are underway to synthesize a more amplified adamantane dendrite with 9 fluoresceins. Moreover, alternative means of linking the fluors to the oligonucleotide probe are being explored. One involves use of probes with a biotin terminus and fluor-labeled avidin. The other makes use of a probe with a reactive terminus to which we plan to link an activated fluor directly once the probe strand is already bound to the target.

Once these goals are met, work will proceed to cytogenetic comparison of the degree of amplification of the Her2/neu gene in normal and malignant tissue culture cell lines and then in fixed pathological tissues.

Xiang-Dong Fu
University of California, San Diego

Cancer Classification Based on MRNA Isoforms

Xiang-Dong Fu[1], Joanne M. Yeakley[1], Jian-Bing Fan[2], Mark Chee[2], David Tarin[3], Michael Gribskov[4], and Michael Zhang[5]

[1]Department of Cellular and Molecular Medicine, University of California, San Diego, [2]Illumina, Inc., San Diego,[3]UCSD Cancer Center, [4]San Diego Supercomputer Center, and [5]Cold Spring Harbor Laboratory

Recent completion of the human genome project has set the stage for understanding network regulation of gene expression in biology. One of the major surprises is the large number of mRNA isoforms generated by alternative splicing (60% of human genes express more than one transcript and this percentage is likely underestimated), which undoubtedly contributes to molecular diversity in complex biological pathways. In our battle against cancer, variations in mRNA isoforms may underlie many previously unrecognized mechanisms for cellular transformation, and therefore may prove valuable for cancer diagnosis and characterization of potential therapeutic targets.

Our project aims at developing a genomic approach to alternative splicing because current techniques for assaying alternative splicing rely on low throughput methods that are not suitable for genome-wide analysis. The technology utilizes the addressable zip-code strategy on fiber optic-based microarrays developed at Illumina. Briefly, DNA oligos are used to cover specific splice junction sequences and each isoform-sepcific oligo is linked to a unique zip-code sequence. The oligos are first hybridized to total RNA and those detecting specific alternative splicing events are selectively amplified by PCR. Individual splicing events are then sorted and quantified on a universal Zipcode array. Using this novel microarray technology, we can detect specific alternative splicing events with unprecedented specificity and sensitivity, reaching to the single cell level in certain cases. Furthermore, semi-quantitative data are obtained from a panel of experimental cell lines and 90% of data points match the results obtained by conventional RT-PCR. Although we have been focusing on technology development in the initial R21 phase, our research has already led to the discovery of a number of striking cell-specific alternative splicing events in our model systems.

In the future, we plan to refine the assay to determine optimal experimental conditions for large scale application of the technology. We are interested in coupling our technology with sample preparation methods, such as immunofractionation of cells and laser capture microscopy. This will allow us to analyze alternative splicing in cell populations associated with distinct cancer stages. In particular, we plan to examine several hundred prostate cancer samples available at the UCSD Cancer Center to explore the utility of variations in alternative splicing for cancer classification. Parallel to our experimental efforts, our collaborators on the team will develop computational tools to aid database construction and analysis.

Suzanne A. W. Fuqua
Baylor College of Medicine
Baylor Breast SPORE

Suzanne A. W. Fuqua

Baylor College of Medicine

The Baylor Breast SPORE Program has permitted the rapid translation and development of new basic research findings into clinical practice. There are five major

projects in the SPORE program. These include: Project 1 (Mechanisms of Tamoxifen Resistance), Project 2 (Heat Shock Proteins and the Pathogenesis of Breast Cancer), Project 3 (Identification of Surrogate Markers in a Tamoxifen-Prevention Trial), Project 4 (Molecular Genetics of Pre-Malignant Breast Disease) and Project 5 (Liposomal Gene Therapy). The SPORE also has two core resources (National Tissue Resource, and the Family Registry Cores). The National Tissue Resource has grown to more than 100,000 tumors. Last year, more than 2,000 specimens were distributed to thirteen investigators at various institutions. The Familial Breast Cancer Registry is an important resource for studies of new breast cancer genes and for identifying patients for prevention trials.

The five research projects have made excellent progress toward our translational objectives. In Project 1 we identified several new estrogen receptor interacting proteins via the yeast two-hybrid screen, and we are characterizing these proteins to see if they can explain tamoxifen-stimulated growth. We also found FKHR as a receptor-interacting protein that has bi-functional activity, inhibiting steroid receptors but stimulating thyroid and retinoic acid receptors. Most interesting, FKHR is found on a locus on chromosome 13 that displays a high frequency of LOH, suggesting the possibility that it could function as a tumor susceptibility gene, with loss of its function resulting in over-activity of the estrogen receptor. In Project 2 we have determined that overexpression of hsp27 confers resistance to doxorubicin and that the key underlying mechanism for resistance is hsp27's ability to inhibit drug-induced apoptosis, and thus to increase the survival of breast cancer cells. Utilizing microarray technologies, we have identified several novel targets of drug resistance, and we hypothesize that hsp27 is a key signaling intermediate.

In Project 3 we have collected tissue samples from a tamoxifen prevention study and they are a unique resource to study the biological mechanisms of how tamoxifen reduces breast cancer in women with premalignant lesions. We are assessing by IHC and microarray for changes in biomarkers between first and second biopsies and between controls (placebo) and tamoxifen-treated patients. In Project 4 we have shown that in hyperplasia from non-cancerous breasts, Loss Of Heterozygosity (LOH) at any given locus is rare. Hyperplastic lesions however showed at least one LOH, suggesting a complex interaction of tumor suppressor genes and environment in the early development of these lesions. In cancerous breasts, many of the precancerous lesions shared LOH with the synchronous cancers, supporting the idea that these premalignant lesions are in fact precursors of invasive breast cancer. RNA based microarray experiments are underway, as well as DNA array studies in collaboration with other SPORE groups to examine the molecular profiles of these early lesions. In Project 5, we are developing adenoviral vectors for liposome mediated gene transfer. In summary there are a number of projects utilizing powerful molecular analysis technologies applied to translational research objectives.

Xiaolian Gao
University of Houston

A Facile and Versatile Method for Parallel Microarray Synthesis

Xiaolian Gao[1], Erdogan Gulari[2], and Xiaochuan Zhou[3]

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Oligonucleotide microarrays are effective decoding and analytical tools for genomic sequences and are useful for an enormously broad range of applications. Therefore, it is highly desirable to have synthesis methods of DNA/RNA chips that are highly flexible in sequence design and also provide high quality and general adoptability. We report herein DNA microarray synthesis based on a flexible biochip technology. Our method simply uses photogenerated acid (PGA) in solution to trigger deprotection of the 5'-OH group in conventional nucleotide phosphoramidite monomers (i.e. PGA gated deprotection), with the rest of the reactions in a synthesis cycle the same as those used for routine synthesis of oligonucleotides. The complete DNA chip synthesis process is accomplished on a regular DNA synthesizer that is coupled with a UV-VIS projection display unit for performing digital photolithography. Using this method, DNA chips containing probes of newly discovered genes can be quickly and easily synthesized at high yields in a conventional laboratorial setting. The PGA gated chemistry is applicable to microarray syntheses of a variety of combinatorial molecules, such as peptides and organic molecules. Our latest progress on RNA chip synthesis will be reported.

H.R. Garner
University of Texas Southwestern Medical Center

Digital Optical Chemistry; A Novel System for the Rapid Fabrication of Custom Oligonucleotide Arrays

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A rapid method for creating custom DNA microarrays is described. Oligonucleotide microarrays are routinely used for resequencing and gene expression studies. In resequencing studies, custom oligonucleotide arrays can be used to discover new single nucleotide polymorphisms, genotype different cancer lines, and aid in determining inherited alleles responsible for familial predisposition toward cancer. Light directed fabrication of oligonucleotide arrays allows for highly parallel synthesis, yielding high feature-density arrays. Ultraviolet light is spatially directed to promote chemical reactions on a solid glass surface. In our approach, a Texas Instruments Digital Light Processor (DLP) is used to direct light, as opposed to traditional photolithographic methods. The DLP consists of ~800,000 digitally controlled microscopic mirrors, which are used to instantaneously create digital masks rather than machining physical

photolithographic masks. This technique allows the researcher to quickly customize arrays for their uses and to quickly modify array designs.

John Gerdes
Xtrana, Incorporated

Binding of nucleic acid to XtraBind permits stable storage and solid phase RT PCR of mRNA for measurement of gene expression

John Gerdes, Jeffrey Marmaro, Shannon Beard, and Craig Sampson

Xtrana, Incorporated, and Denver CO Xtrana, Incorporated, Denver CO

Breast carcinomas are heterogeneous in their biological and clinical behavior. Cell populations where molecular markers will be of most relevance frequently occupy less than 5% of the tissue volume of a tumor or biopsy. Laser Capture Microdissection (LCM) is a method for procuring pure cell populations from specific microscopic regions of tissue sections. Although LCM provides a means of selecting malignant cells of similar morphology, it also presents challenges to accurate analysis of cancer markers due to the relatively small numbers of cells. Sensitive methodologies are needed for measuring low copy mRNA expression levels ideally for several different genes all within the same small number of microdissected cells.

Xtrana is developing a sensitive and precise method for the relative measurement of gene expression following solid phase capture of nucleic acid onto our proprietary Xtra BindTM solid phase matrix. Capture occurs in an Xtra Bind coated PCR tube (Xtra Amp) that is used directly for RT PCR. DNase treatment prior to binding insures that only RNA is captured. We have observed that solid phase RT works best using the AMV RT. Commercially available kits containing protocols using AMV including the Roche Titan 1-step and Promega Access RT PCR kits also provide robust results. Initial investigations have utilized the breast cancer T47D cell line. The optimal cell lysis / Xtra Bind binding buffer is LiCl based.

Solid phase capture offers the advantage of rendering the mRNA stable. Bound mRNA has been stored at room temperature and analyzed 8 weeks later. When interfaced with RT PCR, robust amplification is observed by gel electrophoresis, TaqMan, or other standard detection methods. Extraction, amplification and detection can be performed in the same tube from as few as 10 to as many as 104 cells. The RNA is bound irreversibly and will not elute even following rigorous aqueous washes or following multiple PCR cycles. This property can be exploited for repeated or extended RT PCR reactions from bound RNA from the same specimen.

The interface of Xtra Amp captured mRNA with LCM cell nucleic acid extraction and quantitative fluorescence measurement of gene expression should enable extensive analysis of multiple genes from the same few cells. Briefly, we envision capture of LCM cells onto a cap that fits a standard PCR tube. This tube contains cell

lysis/Dnase/nucleic acid binding buffer and a nucleic acid binding matrix coating the inside of the bottom of the tube (Xtrana's Xtra Amp tubes). By simply mixing, the mRNA is bound inside the tube. The solid phase bound nucleic acid is amplified directly in this tube by RT PCR with real time TaqMan detection.

Roger W. Giese
Bouve College and Barnett Institute

New Methodology for the Analysis of DNA Adducts

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Nearly all of the substances that are classified as human carcinogens cause direct or indirect damage to DNA (DNA adducts). Improved measurements of DNA adducts in human samples may contribute to advances in cancer prevention. Our work is intended to help in filling the following gaps in what current assays provide. (1) Screening and identification of unknown DNA adducts in human samples. For trace adduct screening and identification, we are studying cation labeling mass spectrometry. The purpose of the cation labeling is to establish uniform, high sensitivity detection across a diversity of adduct structures. With current mass spectrometry methodology, sensitivity can depend on the adduct. Towards this goal, we have set up a large laser spot, laser desorption Fourier transform mass spectrometer, and have begun to optimize the detection of cationic tags. Reproducible detection of a model cation tag deposited as a 2 mm diameter spot on the probe target has been achieved at the 0.5 fmol level, with signal persistence for a few shots. This result, especially the high reproducibility, advances the performance of laser desorption mass spectrometry for detecting a favorable small molecule by about 100-fold.

Work on attaching this and other cation tags to DNA adducts is in progress. (2) Comprehensive detection of DNA adducts. For comprehensive adduct detection we are studying chemical labeling with a fluorescent dye, based on the use of a dye-imidazole/carbodiimide reaction that specifically labels a phosphomonoester such as the phosphate group of a deoxynucleotide (Lan, Wang, Giese, Rapid Commun. Mass Spectrom. 13 [1999] 1454). A BODIPY-IMI dye was employed in our initial work. Currently, we are studying xanthamide dyes (prepared recently in our laboratory) because of their enhanced chemical and photostability, and cyanine dyes, because their absorption/emission in the near-infrared region escapes background signals. Recently we have detected a model near-infrared dye in a practical way by injecting 0.5 uL containing 0.5 amol of dye into a capillary electrophoresis laser-induced fluorescence (CE-LIF) instrument.

(3) High sensitivity detection of oxidative sugar DNA adducts. The initial analyte of interest in our sugar oxidation work is phosphoglycolate, which can be released from

DNA by a repair enzyme. We have set up a method involving use of the electrophore-tag reagent, AMACE1 (Lu and Giese, Anal. Chem., 72 [2000] 1798), which detects one picogram (13 fmol) of glycolate in a procedure that terminates with gas chromatography electron capture mass spectrometry (GC-EC-MS). Current work involves the extension of this method to real samples, and broadening to related analytes based on the ability of AMACE1 to label four functional groups (carboxyl, lactone, aldehyde, ketone) in a single procedure. (4) Simultaneous detection of multiple small DNA adducts such as alkyl and hydroxyalkyl. For the detection of small DNA adducts as a class, we are relying on electrophore labeling GC-EC-MS, and studying N7-hydroxyethylguanine as a representative analyte. Our current methodology detects 10 picograms of this compound, and our next analytical goal is to get closer to the detection limit of 10 femtograms for a diluted standard of the final derivatization product. Detecting a diversity of adducts of this type in a single procedure is our long-term goal. Thus, these emerging CE-LIF and MS techniques potentially will advance the measurement of DNA adducts in human samples.

V. Golovlev
Sci-tec, Inc.

DNA Micro-Array Magnetic Reader

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Fast growing applications of microarray in biomedical fields has created strong demand of affordable and reliable methods for preparing and reading DNA micro-arrays. To address this demand, Sci-Tec, Inc. is currently working on development of ultra-sensitive and cost-efficient micro-array system for bio-medical applications. The core of the approach is to detect metal clusters attached by a DNA molecule to the surface of magnetic media (magnetic disk). The spatial location of hybridized probe-target complexes can be determined by monitoring magnetic properties of the surface. The information about location of the bounded DNA then can be used to identify the presence of target DNAs with specific sequence. An important feature of this approach is that the DNA detection is based on the attachment of relatively large tagging particles. Since the detection signal is proportional to the amount of tagging material, the sensitivity of DNA detection can be improved by controlling the size of the particles.

With our approach, magnetic detection can be employed using either magnetic or non-magnetic particles for DNA labeling. To detect the change of magnetic field triggered by the binding reaction on the surface we have developed a reader system based on a floppy drive of a personal computer (PC). An electronic circuitry was modified to acquire high-resolution analog signal from the magnetic head of the drive. The head, controlled by a computer, scans the surface of the disk and produces 2D magnetic map of the array surface.

Resolution, i.e., system's ability to distinguish large number of closely located spots, is very important issue for high-density micro-arrays. In order to assure our reader can be used with high-density micro-arrays we have tested system's performance by preparing and reading array with the size of spots from 1500 to 30 um. Reliable detection was achieved down to the size of probe spots of 60 um and separation between two neighboring spots of 100 um. The experimental results show the system has the potential to detect up to 45,000 DNA probes on a single 3.5 diskette.

Currently the work is in progress for optimizing and using the disk array system for gene mutation analysis. Hybridization experiments are pursued using a set of synthetic probe-target oligos designed from the locus HSU62962 of the IL6: Interleukin 6 (7p21). The set of probes will be expanded in the future to focus on the diagnostics of mutations relevant to different cancers.

Jeffrey Griffith
University of New Mexico School of Medicine
Chemiluminescent Measurement of Telomere DNA Length in Prostate Biopsy

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Telomeres are protein-DNA complexes located at the ends of linear chromosomes that protect them from degradation, recombination and end-to-end fusions. Telomeres are progressively shortened every time a cell divides--ultimately leading to genomic instability and phenotypic variability. 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 death and disease recurrence ($p<0.0001$, $p<0.0001$, respectively). However, our initial assay for telomere DNA content lacked the sensitivity needed to analyze biopsy material, required 32P-labelled probes, and took 7-10 days, making it poorly suited to large-scale investigations and clinical settings. The goal of the initial phase of this project was to develop an assay for telomere DNA content that had the sensitivity needed for use with biopsy materials and was suited for large-scale investigations and clinical settings. DNA was purified from archival samples or obtained commercially and quantitated using a commercially available fluorescent reagent, PicoGreen. DNA was denatured, fixed to a positively charged membrane and hybridized with a telomere specific oligonucleotide (5TTAGGG3)4 labeled with fluorescein.

Michael Gruidl
H. Lee Moffitt Cancer Center and Research Institute at USF
The Biomarker Development Laboratory at Moffit (BeDLAM)

Michael Gruid

H. Lee Moffitt Cancer Center and Research Institute at USF

This group of ten investigators focuses on developing assays to detect preclinical lung cancer proteins and altered DNA in body fluids. They have already identified one potential biomarker, expression of hnRNP A2/B1 in exfoliated airway epithelial cells, which is currently in clinical trials. New markers from collaborating laboratories at Moffitt are being developed, refined and compared on common paired tumor/normal specimens from the Moffitt core tissue bank. These biomarkers include: a difucosylated ceramide, lacto-N-fucopentose III; markers of the TGF-beta signaling pathway, TGF-beta receptor Type II, SMAD 2, SMAD 4, and SMAD 7; and markers of tumor suppressor genes silenced by promoter methylation and by allelic loss. Technical approaches include Enzyme-linked immunosorbent assays, Western blot analysis, methylation specific PCR, immunostaining, thin Layer chromatography, automated DNA sequencing and laser-scanning immunofluorescence. These panels of assays are being developed as complementary technologies to helical CT detection of pre-clinical lung cancer. Promising biomarker assays from this project will be applied to archived sputum specimens collected during the ongoing Moffitt helical CT lung cancer screening trial "Markers of Transformation in airways Epithelial Cells from a Cohort of Obstructed smokers and Former Smokers". This archive will provide preclinical material with subsequent known cancer outcome for final biomarker case-control assay. The final assay will be conducted on a high-throughput screening platform currently under development by a Collaborative Research and Development Agreement industrial partner. Comparisons of additional biomarkers on these specimens will be facilitated through interactions with the Lung Cancer SPORE programs at Johns Hopkins, University of Colorado and MD Anderson/Texas SW.

Baochuan Guo
Cleveland State University

MALDI-TOF Technology For Molecular Analysis of Cancer

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Point mutations are one of the most common genetic changes leading to cancer and therefore the detection of point mutations is essential to cancer research and diagnosis. Solid tumor specimens often contain a significant number of normal cells and the mutant cells present in specimens may be less than 1%. The specimens from other sources may contain an even smaller percentage of mutant alleles. Thus, assays for tumor detection need to be highly sensitive and specific, such that mutated alleles can be readily detected in a large background of wild-type alleles. Moreover, cancers arise from mutations in multiple genes and therefore, a good assay should allow the simultaneous identification of multiple mutated genes.

This primary focus of our proposed research is the development of mass spectrometric based technologies for automated, multiplexed, high-throughput, sensitive, and specific detection of a small population of point mutation tumor cells in a large background of wild-type cells. The technology developed in this work consists of three major steps. First, the clinical DNA samples are amplified using the peptide nucleic analogues (PNA) directed PCR clamping reactions in which mutant DNA are preferentially amplified; second, the PCR amplified DNA fragments are extended through mini-sequencing to generate diagnostic products; and third, diagnostic products are identified using matrix-assisted-laser-desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and therefore the presence and nature of mutations are determined. Our preliminary results have demonstrated that this approach could identify mutant alleles in the presence of over 1000-fold excess of normal alleles. This preliminary work was performed using both tumor and bronchoalveolar lavage fluid (BAL) specimens from lung cancer patients. Thus, the next logical step is to develop this method and to explore its potential in cancer research and detection. Two experimental goals will be achieved in this project. First, we will prove the feasibility of this new technology to identify various cancer-causing point mutations using both single and multiplexed assays. Second, we will develop the proven assays for the detection of the hotspot point mutations in both k-ras and p53, two of the most important genes related to cancers.

Bassem R. Haddad
Georgetown University Medical Center

Molecular Cytogenetic Evaluation of Mammary Epithelial Cells in Nipple Aspirate Fluid

Bassem R. Haddad, Marie Pennanen, Janice D. Rone, Luciane R. Cavalli, Bruce Trock, and Robert B. Dickson

Lombardi Cancer Center, Georgetown University Medical Center

Objectives: Our project involves the development of a new, non-invasive approach for early detection of breast cancer, based on chromosomal analysis of mammary epithelial cells shed into the nipple aspirate fluid (NAF). Methods: The proposed research involves the use of a state-of-the-art molecular cytogenetic technique, comparative genomic hybridization (CGH), to detect chromosomal aberrations in NAF-derived epithelial cells that have been expanded in short term cell culture. This approach circumvents the two major limitations to conducting cytogenetic analysis in NAF-derived cells: (1) the inability to obtain good quality metaphase preparations from NAF cells, and (2) the low cellularity of NAF samples. The DNA isolated from the short term culture with expanded cell number (250-500 cells) is amplified using a universal DNA amplification protocol, degenerate oligonucleotide primed PCR (DOP-PCR), prior to CGH evaluation.

Methods: The proposed research involves the use of a state-of-the-art molecular cytogenetic technique, comparative genomic hybridization (CGH), to detect chromosomal aberrations in NAF-derived epithelial cells that have been expanded in

short term cell culture. This approach circumvents the two major limitations to conducting cytogenetic analysis in NAF-derived cells: (1) the inability to obtain good quality metaphase preparations from NAF cells, and (2) the low cellularity of NAF samples. The DNA isolated from the short term culture with expanded cell number (250-500 cells) is amplified using a universal DNA amplification protocol, degenerate oligonucleotide primed PCR (DOP-PCR), prior to CGH evaluation.

Results to date: Here we report on our preliminary findings with the analysis of 50 NAF samples. We will discuss the advantages of this approach and highlight the major hurdles encountered. Our preliminary results clearly support the feasibility of this approach. However, the results also indicate that future development of this method heavily rely on the ability to improve the number of mammary cells available for testing. Efforts towards that end are underway in our laboratory.

Plans for the Future: One very promising, new approach to overcoming low NAF cellularity has recently been designed and tested: Breast Ductal Lavage. It permits the collection of a large number of mammary cells (in the thousands) by introducing a very small catheter to the breast duct and performing a "ductal lavage" with saline. This procedure is now available at our institution. At the present time, all collected fluid is sent for cytologic evaluation. Once the procedure is validated at our institution, we plan to use an aliquot of the fluid to perform our genetic evaluation (CGH). Breast ductal lavage promises to provide a much higher success rate than nipple aspiration.

Dorothee Herlyn
The Wistar Institute

Molecular cloning of HLA Class II tumor antigens

Dorothee Herlyn, Rajasekharan Somasundaram, and Kapaettu Satyamoorthy

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Our major goal is to develop a novel technology to identify HLA class II tumor antigens as potential vaccines for cancer patients. These vaccines may induce a T helper (Th) response important for the activation of cytolytic T lymphocytes and other effector cells of the innate immune system in patients. To clone HLA class II-dependent Th tumor antigens, tumor cell cDNA libraries are expressed by the phages, followed by library phage presentation to Th cells by antigen-presenting cells and identification of the relevant Th antigen in cytokine release assay. This approach has numerous potential advantages over existing approaches to class II antigen cloning or biochemical peptide isolation. To develop this approach, we have available a unique model system including Th cells against tetanus toxoid (TT) and a cDNA fragment encoding the TT-associated Th epitope. Using this model system during the R21 phase of this study, we have: i. developed phage vector with TT insert; ii. expressed TT in phages; iii. demonstrated that control phages do not act as superantigens in Th cell stimulation assays; iv. shown that anti TT Th cells specifically proliferate after stimulation with TT-phages presented to

the Th cells by autologous Epstein Barr virus-transformed B cells; and v. determined that the sensitivity of Th cell stimulation by TT-phages is one TT-phage in fifty irrelevant phages. In future studies (R33) the described technology will be used to clone melanoma and colorectal cancer-associated Th antigens using available Th lines and clones.

Jonathan Jarvik
Carnegie Mellon University

High Throughput Proteomics Using CD-Tagging

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A true understanding of the biology of any cell requires knowledge of all of its proteins and what they do. This knowledge would include a catalog of the complete set of expressed proteins and a description of their locations, abundances, enzymatic activities and interactions with other molecules in the cell and in the extracellular environment. Such a description would also include information about how each protein changes in abundance, location, and structure as the cell traverses the cell cycle and as it responds to changes in its external or internal environment. In other words, one would like to "see" each member of the proteome and follow its various activities at the molecular and cellular levels.

CD-tagging provides a unique means to gain the kind of knowledge referred to above.. With CD-tagging, a special CD-cassette is inserted into genomic DNA. When the insertion occurs into the proper orientation in an intron in an expressed gene, the result is the addition of a unique guest exon to the mRNA and the addition of a unique guest peptide to the encoded protein. By visualizing this guest peptide, we are able to both visualize and isolate the tagged protein.

We have used a transcription-free stealth retroviral vector to deliver CD-tags to the genomes of NIH3T3 fibroblasts. The vector carries two CD-cassettes one with a guest exon encoding GFP so that the tagged proteins can be detected and imaged in living cells, and the other with a guest exon encoding an epitope tag so that the proteins can be purified and characterized. Clones tagged in numerous cellular locations have been recovered and are presently under close molecular and cell biological analysis. Our results encourage us to scale up our activities with the goal of creating an accessioned library of cell clones tagged in every gene whose product is expressed in sufficient quantity to be detected by high resolution fluorescence microscopy.

Daniel G. Jay
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A High-Throughput Screen for Cancer Cell Invasiveness using Fluorophore-Assisted Light Inactivation

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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 Fluorophore-Assisted Laser Inactivation (FALI) to inactivate proteins of interest in cancer cells to address their roles in invasiveness. FALI 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 fluorophore. We have coupled FALI to a transwell invasiveness assay to allow for high throughput screening. We have tested the 1 integrin (a previously?efficacy of this screen using antibodies against validated target for invasiveness). We showed tested FALI using a 20-antibody screen and showed that one of these recognizes a protein that has a role in invasion of HT-1080 fibrosarcoma cells. We are now screening 1,000 single chain Fv (ScFvs) molecules from Xerion Pharmaceuticals directed randomly against antigens expressed by HT-1080 cells.

Progress of this screen will be reported. Major advantages of this approach include: 1) the ability to target proteins that have essential roles disease-relevant processes; 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). 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.

David L. Jaye
Emory University School of Medicine

Phage Display and Prostate Neoplasia Progression

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The goal of this project is to utilize a novel technology, random peptide phage display, to identify and characterize peptides that differentiate phases of progression of prostate neoplasia by identifying molecular alterations in tissues. In the developmental phase of these studies that is just underway, the technology is being advanced for use in identification of peptides that differentiate benign from malignant tissues, using human prostate cancer as a model system. To do this, new affinity selection procedures are

being developed. To detect peptide-bearing phage particles when bound to both formalin-fixed, paraffin-embedded, and fresh tissues, novel histochemical techniques are being defined. The identified phage will be tested on a large series of human prostate cancer cases to confirm binding specificity and reproducibility in histochemical studies. The developed methodologies and first generation phage reagents will then be utilized in a pilot application in which phage will be identified that bind to and discriminate between the precursor of prostate cancer (prostatic intraepithelial neoplasia) and prostate cancer, on the one hand, and organ-confined prostate cancer and metastatic cancer on the other. The identified phage bearing specific peptides from these experiments will be tested in well-defined clinicopathologic studies on a large series of cases to assess their value as predictors of disease progression (from pre-cancer to cancer, and from cancer to metastatic disease). Important correlations will be made with clinical and pathologic data that will be available on each of the cases used in these studies.

Tanya Kanigan
Beth Israel Deaconess Medical Center and Harvard Medical School

Androgen Receptor Bio-Chips: Yeast Based Micro-Bioassays for Serum Androgens in Men with Prostate Cancer

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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 from patients with prostate cancer. In contrast to the immunoassays currently used to measure steroid hormones, yeast -based AR micro-bioassays are designed to assess the receptor response to all of the available ligand in a complex biological sample. In healthy males and in prostate cancer patients, AR bioassay serum androgen is a parameter distinct from total and free serum testosterone. We have shown, in an established animal model for prostate cancer, that AR bioassay serum androgen is a better predictor than serum testosterone for the response of a prostate tumor (LNCaP) to dietary phytoestrogens. We have also demonstrated that the AR micro-bioassay can be used to identify novel competing AR ligands of pharmacological interest.

In parallel, we have demonstrated the feasibility of performing these assays in silicon through-hole microarrays. As we scale-up this technology, these microarrays will be incorporated into a platform capable of performing AR bioassays on hundreds of sera samples simultaneously, with sample volumes of less than 100 nL.

Francis G. Kern
Southern Research Institute

Hormonal Resistance, Mechanisms and Reversal

Francis G. Kern

Southern Research Institute Birmingham, AL

Antiestrogen treatment is a useful therapy for many breast cancer patients but an invariable occurrence in tamoxifen-treated patients with metastatic disease and a far too frequent occurrence in patients receiving tamoxifen adjuvant therapy is the outgrowth of populations of resistant cells. Signal transduction resulting from overexpression of growth factors or growth factor receptors can either mimic the effects of estrogen or bypass a breast tumor cells need for estrogen for growth and is therefore a possible cause of antiestrogen resistance. We have found that overexpression of members of the fibroblast growth factor family increases the rate of cellular proliferation when estrogen receptor positive breast cancer cells are cultured in estrogen-depleted or antiestrogen containing media.

Overexpression of FGFs also results in cells acquiring the ability to rapidly form progressively growing tumors in ovariectomized athymic nude mice without estrogen supplementation. Tumors also develop in mice treated with tamoxifen, aromatase inhibitors or the pure antiestrogen ICI 182,780, suggesting that FGF signaling provides an alternative growth signaling pathway that bypasses the need for a growth pathway mediated by estrogen receptor alpha activation. Tumors still develop in tamoxifen-treated animals when autocrine or intracrine FGF signaling within the breast cancer cells is abrogated through expression of a dominant negative FGF receptor. This suggests that FGF- mediated neoangiogenesis may increase the agonistic properties of tamoxifen by decreasing the rate of apoptosis within the tumor. Formation of tumors in tamoxifen-treated animals when the angiogenic growth factor VEGF is overexpressed also supports this hypothesis.

Together these results raise the possibility that inhibition of growth factor signaling and treatment with angiogenesis inhibitors may offer a means of restoring sensitivity to antiestrogens. Transfection studies with cDNAs encoding dominant-negative or constitutively active mutants of receptors and potential key intermediates in various signal transduction pathways are being employed to elucidate those operative within breast cancer cells that are causative of the antiestrogen resistance mediated by FGFs and other growth factors. These studies include the use of novel expression vectors we especially designed to maintain long-term stable expression of specifically modified doxycycline-regulated transactivator fusion proteins to permit the expression of these cDNAs in an inducible manner. They should assist in the identification of targets for the development of novel therapeutic intervention strategies aimed at restoring antiestrogen sensitivity. Neoangiogenesis resulting from FGF or VEGF overexpression also facilitates tumor cell dissemination to distant organs where they can be detected as micrometastases but fail to develop into tumor nodules. The laboratory is therefore also

attempting to identify what further changes in gene expression in FGF or VEGF transfected cells will allow them to overcome tumor cell dormancy and acquire a greater metastatic potential. Finally, the FGF and VEGF transfected cell lines are also being used as preclinical animal models to assess the antitumorigenic or antimetastatic effects of agents capable of inhibiting neoangiogenesis or growth factor signal transduction.

Matthias H. Kraus
University of Alabama at Birmingham
Validation of Candidate Molecular Targets Identified by DNA Micro-Array Analysis in Clini-cal Breast Cancer

Matthias H. Kraus and J. Michael Ruppert

University of Alabama at Birmingham, Birmingham, AL

Obtaining informative expression data using clinical tissue specimens requires quantitative detection of gene expression in vivo. Such detection is limited by cellular heterogeneity, degradation of resi-dent macromolecules, tissue quantity or sensitivity of detection procedures. To overcome some of these limitations we plan using both cRNA *in situ* hybridization (ISH) and real-time, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for determining transcript levels of in-dividual candidate molecules in tissues. Combining near single cell resolution of the former and high sensitivity and quantitation of the latter technique we anticipate useful mRNA expression in-formation in settings of clinical trial studies. This in turn appears critical in establishing genuine tar-gets of tumor pathogenesis, treatment response or therapeutic refractoriness. Our interest focuses on downstream targets of oncogenic transcription factors MYC and GKLF that are frequently overex-pressed in primary breast cancer. Candidate targets will be selected following DNA array analysis of recombinant epithelial model systems, and subjected to expression analysis in clinical breast cancer.

Klaus Kunze
Superior MicroPowders
Small Particle Phosphor Powders for Immunoassays

Klaus Kunze and Mark Hampden-Smith

Superior MicroPowders, Albuquerque, NM

Superior MicroPowders, in collaboration with STC Technologies, is currently developing a spectrum of novel phosphor powders for a reporter technology based on up-converting phosphor materials for use in phosphorescent immunoassays (PIAs). 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 unique spray powder manufacturing technology allows for production of phosphors with control over crystalline phase, morphology, particle size, and particle size distribution by spray routes. The up-conversion efficiency is strongly dependent upon the choice of host materials, activator and sensitizer concentrations, particle geometry, particle size and particle size distribution. Using YF3:Er,Yb as an example for a green emitting IR up-converting phosphor we are currently exploring the processing conditions required to produce phase pure nano-sized phosphor powders with a narrow particle distribution that show optimum emission intensities. Different routes to the formation of phase pure materials as well as the influence of process conditions on the materials characteristics will be presented.

Kit S. Lam
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Identification of cancer cell surface binding ligands and characterization

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Using a split-synthesis method, we generate random peptide-bead libraries such that each bead displays only one peptide entity. When this "one-bead one-compound" peptide-bead library (106) is mixed with intact cancer cells, the surface of some of the beads is covered by a monolayer of cancer cells. These beads are then isolated for sequence determination. Using this whole-cell screening approach, we succeed in the identification of peptide ligands that bind to the cell surface of human B- and T-cell lymphoma cell lines. Some of these peptides bind preferentially to the malignant cells but not normal peripheral blood mononuclear cells. To analyse the binding specificities of the identified ligands against a large number of cancer specimens, we developed a high-throughput peptide-microarray method. In this method, peptides are chemically conjugated onto a glass slide in a microarray format, and cell suspension derived from cancer biopsy specimens are layered over the microarray. After gentle washing, binding specificity of the peptide ligands against patients' normal and cancer cells can be determined by staining the slide with Geimsa stain. Furthermore, with the appropriate fluorescent-labelled antibodies, the biochemical effect of each of the ligands on the cancer cells can be determined by viewing the cell-bound peptide microarray under a fluorescent microscope.

Steve Lesko
Cell Works, Inc.

Blood Biopsy for epithelial cancer cells based on circulating cancer cell tests

Paul O.P. Tso, Steve Lesko, Vivian Lauderdale, Steven Zoha, and Karen Ohara

Cell Works, Inc., Baltimore, MD 21224

The importance of early detection of cancer recurrence after treatment of a primary tumor, early diagnosis of metastasis, and early information about the responsiveness to systemic chemical/hormonal therapies, are well recognized for management of cancer patients. By spiking cultured cancer cells from more than thirty cancer cell lines of Prostate, Breast, Colon, Gastric, Liver, Kidney cancers, etc. into 20 ml of human blood, Cell Works has established a universal procedure, the Circulating Cancer Cell (CCC) Test to detect circulating epithelial cancers. This procedure utilizes a double gradient sedimentation for the removal of most RBC and WBC as well as magnetic cell sorting for the additional removal of WBC before spreading the cancer cells onto a slide utilizing a cytospin apparatus. The fixed cells on the slide are then stained with various specific molecular probes, with selected fluorescent dyes attached. These cells are automatically scanned with an award winning spectroscopic microscope system, first in low magnification, where the fluorescent digital image is captured at a resolution of 0.2 um using multiple excitation/emission wavelengths, then at higher resolution for further analysis. The system has automatic adjustment of exposure, focus and other parameters required for proper image acquisition and analysis to identify cancer cells and markers on the basis of intensity and blob analysis. Image storage and records are automatically kept, maintained on a case by case basis for production of print outs and reports. This test can routinely provide five types of basic information about individual cancer cells found in the blood sample:

1 The number of epithelial cells by the use of a cocktail of 4 types of monoclonal antibody probes for cytokeratins and tumor specific surface antigens. The recovery rate is usually 50-80%. Sensitivity experiments, where 10 cancer cells are added to 20 ml of blood (which contains 120-200 million WBC), show that at least 5 cancer cells would be detected. Preliminary data on blood samples from 13 prostate cancer patients indicate that if there are 3000 circulating cancer cells in the whole body, several cancer cells would be detected in the 20 ml of sample.

2 These epithelial cells in the blood have abnormally high DNA content when compared to the normally diploid WBC of the same patient on the same slide. The ratio of CCC/WBC DNA content is in the range of 1.5-3.0, indicative of aneuploidy and polyploidy, which are indications of neoplasia. These epithelial cells have an abnormally large two dimensional nuclear area (flattened volume).

3 Absence or the presence of certain proteins such as androgen receptors, estrogen receptors, progesterone receptors, Vitamin D receptors, etc. can be analyzed, which provides valuable information for patient therapy design and management. Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from this Blood Biopsy will provide valuable, previously unavailable data to the attending physicians of these patients.

4 Absence or the presence of certain proteins such as androgen receptors, estrogen receptors, progesterone receptors, Vitamin D receptors, etc. can be analyzed, which provides valuable information for patient therapy design and management. Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from this Blood Biopsy will provide valuable, previously unavailable data to the attending physicians of these patients.

Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from Blood Biopsy will provide valuable, heretofore, unavailable data to the attending physicians of these patients. Active clinical studies in providing valuable data on how to utilize this technology of CCCT are in progress.

In addition, the CCCT will provide very valuable information for basic oncology research about leakage of cancer cells from the primary tumors and the secondary tumors into circulation as well as the fate of these circulating cancer cells in the body. In a recent publication (Cancer, 88: 2787-2795, 2000), we provide evidence which strongly suggests that the circulating cancer cells can divide and multiply in the blood, and this information was not known before. These circulating microtumors can be the initial bridgehead for metastasis.

Richard M. Levenson
CRI, Inc.

An agile lamp for practical, brightfield multispectral microscopy

Paul J. Cronin, Clifford C. Hoyt, Peter J. Miller, and Richard M. Levenson

Instrument Division, CRI, Inc., Woburn, MA 01801

Objectives: Spectral imaging involves the measurement of an optical spectrum at every pixel of an image and can be an important tool for molecular pathology; uses include multicolor fluorescence and spectral karyotyping. While fluorescence represents the current method of choice for detecting and quantitating molecular signals *in situ* and in array-based assays, there are considerable advantages to performing multiplexed signal acquisition in brightfield mode using spectral imaging. However, available technologies, such as Fourier-transform interferometry and tunable filters, are typically expensive, bulky, or slow, and can provide poor spatial resolution. In addition, a great deal of computer processing is needed to extract information from the raw data: interferometers must perform FFTs on megapixel data sets, and all approaches involve intensive calculations of spectral indices in order to classify or analyze the scene into its components.

TMethods: We report on a novel agile lamp for imaging which produces illumination having any desired spectral flux distribution ranging from pure spectral bands of 10 nm or less to precisely-tailored, complex polychromatic functions. This lamp, together with a CCD camera, is suitable for most spectral imaging programs, and by enabling one to directly image the scene in the spectral measure of interest, the spectral processing is done optically, eliminating the need for intensive computer processing. Using three exposures with suitable polychromatic illumination functions, one can directly image a sample in CIE colorimetric space, under any desired illuminant or at any color temperature. Using matched filtering, one can obtain full information from all relevant spectral bands in a handful of exposures with optimal signal-to-noise. The device will last thousands of hours and will maintain spectral stability. It is designed to be

affordable, compact, and rugged, and will mount readily to standard microscopes. Finally, as no interferometer or filter optics are involved, photon efficiency is essentially 100 percent.

Results to date: CRI has assembled a prototype and tested its spectral resolving power and imaging speed. Spectral range is from 420 to 700 nm, and spectral illuminants can be switched in approximately 1 ms. Coupling of the light into a fiber and launching the illumination into a microscope has been accomplished. As proof-of-principle, it will be used to acquire spectra from samples with known and well characterized spectral properties, as well as from complex scenes such as H&E-stained breast cancer specimens.

Plans for the future: Matched filtering mathematical algorithms will be adapted in order to shape the spectral output of the device. This feature will be tested for use in multicolor immunohistochemistry and brightfield in-situ hybridization. It will also be adapted to automatically direct laser-capture microdissection for MALDI/MS-based proteomics using feature classification algorithms with machine-learning capabilities.

Paul M. Lizardi
Yale University School of Medicine

Messenger RNA profiling by single molecule counting

Stefan Hamann[1], John Leamon[1], David C. Ward[2], Deborah Dillon[1], and Paul M. Lizardi[1]

[1]Dept. of Pathology, and [2]Dept of Genetics, Yale University School of Medicine, New Haven, CT

The aim of this project is to develop protocols that generate precise gene expression profiles for 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 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 15-fold greater signal as compared to a standard protocol where probes are labeled by direct incorporation. The signals in the microarray are analyzed in two stages, first using a standard scanner, and then using a 60X objective in a microscope with an X-Y stage. In the second stage, the microscope images only the weakest spots by using intensity and coordinate information from the

first scan. We then use image analysis software to count signals arising from single molecule hybridization events in the lowest intensity spots of the microarray. Expression ratios from the high intensity spots are then combined with digital ratios derived from the single molecule counting data, to generate a complete expression profile for the microarray experiment.

The method has been validated using RNA from tissue culture cells. For small RNA samples (less than 50,000 cells) the coefficient of variation of the ratios of duplicate spots is above 20% for one third of the data points, and below 20% for two thirds. We are currently assessing is this data scatter can be reduced by using a recently published array printing chemistry based on a buffer with betaine. In the near future, we plan to use these methods for the expression analysis of tissue samples containing a few hundred cells.

Reuben Lotan
The University of Texas, M.D. Anderson Cancer Center

Biomarkers for Upper Aerodigestive Tract Lesions

Reuben Lotan

The University of Texas, M.D. Anderson Cancer Center

To identify and understand early events in lung carcinogenesis, we used a cDNA array to screen for genes that are expressed differentially in normal human bronchial epithelial (NHBE) cells and a tumorigenic cell line (1170-I) derived from immortalized HBE cells after exposure to cigarette smoke condensate in vivo. Among these genes, we have identified the S100A2 gene, which encodes a nuclear calcium-binding protein, as being down-regulated in the 1170-I cells. Because this gene has been implicated as a tumor suppressor in breast cancer, we examined its potential role as a tumor suppressor in lung carcinogenesis. Levels of S100A2 transcript and protein, which were high in NHBE cells, decreased by up to 50% in immortalized HBE cells (BEAS-2B and 1799) and to low to nearly undetectable levels in transformed (1198) and tumorigenic (1170-I) HBE cells. Furthermore, S100A2 mRNA and protein were undetectable in 8 and expressed at a reduced level in 3 of 11 non-small cell lung cancer (NSCLC) cell lines. Positive immunohistochemical staining of S100A2 was detected in the majority (75% to 83%) of normal and hyperplastic lung tissues, whereas it was detected in only ‹10% of metaplastic lung tissues, squamous cell- and adeno- carcinoma. Treatment of 1170-I HBE cells and NSCLC cells with 5-aza-2?-deoxycytidine, resulted in partial restoration of S100A2 expression in 7 of 8 cell lines. Our results suggest that S100A2 expression is suppressed early during lung carcinogenesis possibly by hypermethylation of its promoter, and its loss may be a contributing factor in lung cancer development or a biomarker of early changes in this process.

Arun Majumdar
University of California

Optomechanical Chips for Molecular Profiling of Cancer

Guanghua Wu[1], Chris Dames[1], Katherine Dunphy[1], Min Yue[1], Karolyn Hansen[2], Thomas Thundat[2], Ram Datar[3], Richard Cote[3], and Arun Majumdar[1]

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It is becoming increasingly evident that high-throughput identification and quantitation of a large number of biological molecules is important for generating a molecular profile that is critical in diagnosis, monitoring, and prognostic evaluation of complex diseases such as cancer. For genetic analysis, commercially available nucleic acid microarrays allow sensitive identification of thousands of DNA sequences simultaneously. For protein analysis, which is directly relevant for disease detection, high-throughput diagnostics has, however, remained a challenge. Multiplexed protein analysis techniques currently used can be broadly divided into the four different categories, namely: (i) radioactive, chemiluminescent, or fluorescent reporting of antigen-antibody binding; (ii) time-of-flight mass spectroscopy; (iii) electrophoretic separation; and (iv) detection of changes in surface properties due to antigen-antibody binding. While they all have their individual strengths, they currently suffer either from the inability to identify or quantitate proteins, or non-specific binding of a serum analyte to the sensor surface. Truly universal label-free biosensors for sensitive and specific detection of protein analytes in a high-throughput fashion are still to become a reality.

Recent work has shown that when specific biomolecular binding occurs on one surface of a microcantilever beam, intermolecular nanomechanics bend the cantilever, which can be optically detected. While this label-free technique readily lends itself to formation of microcantilever arrays, what has remained unclear is the technologically critical issue of whether it is sufficiently specific and sensitive to detect disease-related proteins at clinically relevant conditions and concentrations. Using microcantilevers of different geometries we have been able to detect two forms of prostate specific antigen (PSA) over a wide range of clinically relevant concentrations and conditions found in sera. This has allowed us to develop a chemomechanical database (surface energy change versus analyte concentration) for PSA. Similar work is also in progress to first detect and subsequently form a chemomechanical database for human chorionic gonadotropin (hCG). Elevated levels of hCG are seen mainly in sera of patients with trophoblastic tumors, but have also been reported in breast, ovarian, gastrointestinal and lung cancers. In addition, experiments are currently being performed to detect DNA-protein binding as well as DNA base pair mismatches.

While chemomechanical databases are being formed for individual tumor markers, we are also in the process of developing microcantilever arrays chips for quantitative detection of multiple analytes simultaneously. The nanomechanical design of the chips are based on the chemomechanical response of the analytes, while they are being fabricated using standard photolithography-based micromachining techniques. The

cantilevers can be designed in a way that over 100 nm of deflection can be generated over the range of clinically relevant concentrations, thus allowing optical detection using diffractive optics. Diffractive optics allows simultaneous detection of the deflection of multiple cantilevers, thus facilitating high-throughput analysis. There are several challenges that are being addressed during chip development, namely: (i) residual stresses in cantilevers and their effect on initial bending; (ii) effect of initial bending on diffraction-based optical detection; (iii) microfluidics design of the chip for multiple functionalization and detection; (iv) temperature control of microchip system.

Sergei R. Malkhosyan
The Burnham Institute

Comparative Hybridization of AP-PCR Arrays, New Method for Analysis of Single Copy Chromosomal Losses and Gains in Cancer Cell Genome

Sergei R. Malkhosyan andSvetlana Baranovskaya

The Burnham Institute, La Jolla, CA

The goal of this project is to develop a new method, Comparative Hybridization of AP-PCR Arrays (CHAPA), for detection of tumor-specific gains and losses of genetic material at multiple loci throughout the cancer cell genome in a single experiment. The CHAPA method combines two techniques: Arbitrarily Primed PCR (AP-PCR) and DNA array technology. AP-PCR is a PCR-based technique that uses a single arbitrarily chosen primer, with several initial cycles performed at low stringency. Because the primer anneals at multiple sites, the technique generates a large number of anonymous PCR products. In the CHAPA approach, AP-PCR is used to obtain unbiased reduced complexity genomic representations for DNA array hybridization. Individual DNA fragments of an AP-PCR product are arrayed on a glass slide. This array is hybridized with a mixture of AP-PCR products which are obtained from tumor and matched normal samples and labeled with different fluorescent dyes. The ratio of hybridization signals at a particular array element between two differently labeled probes reflects the abundance of the genomic sequence corresponding to this array element in normal and tumor samples. Because the AP-PCR representation is produced by a single-step PCR amplification using a single oligonucleotide primer, the technique is very simple, reproducible, reliable, and quantitative. A reduction of the hybridization probe complexity by AP-PCR makes the CHAPA method more sensitive as compared to techniques which use total genomic DNA for array hybridization and allows analyzing minute amounts of the genomic DNA for gains and losses of genetic material.

The CHAPA method combines two techniques: Arbitrarily Primed PCR (AP-PCR) and DNA array technology. AP-PCR is a PCR-based technique that uses a single arbitrarily chosen primer, with several initial cycles performed at low stringency. Because the primer anneals at multiple sites, the technique generates a large number of anonymous PCR products. In the CHAPA approach, AP-PCR is used to obtain unbiased reduced complexity genomic representations for DNA array hybridization. Individual DNA

fragments of an AP-PCR product are arrayed on a glass slide. This array is hybridized with a mixture of AP-PCR products which are obtained from tumor and matched normal samples and labeled with different fluorescent dyes. The ratio of hybridization signals at a particular array element between two differently labeled probes reflects the abundance of the genomic sequence corresponding to this array element in normal and tumor samples. Because the AP-PCR representation is produced by a single-step PCR amplification using a single oligonucleotide primer, the technique is very simple, reproducible, reliable, and quantitative. A reduction of the hybridization probe complexity by AP-PCR makes the CHAPA method more sensitive as compared to techniques which use total genomic DNA for array hybridization and allows analyzing minute amounts of the genomic DNA for gains and losses of genetic material.

Here we report the development of the first version of the CHAPA method, which can detect single chromosomal sequence copy number changes in the tumor cell genome at about 1,000 genomic loci in a single hybridization experiment. In its final form, the CHAPA technique will provide fast, automatic analysis of cancer-specific chromosomal loci copy number imbalances with a resolution of less than 1 Mbp. This high-density molecular karyotyping will be used as a tool for molecular profiling of cancer and can facilitate the identification of novel cancer genes.

Michael McClelland
Sidney Kimmel Cancer Center

Arbitrarily primed PCR as a tool to detect a subset of rare transcripts

John Welsh, Gaelle Rondeau, Takuya Higashiyama, Yipeng Wang, and Michael McClelland

Sidney Kimmel Cancer Center

When a primer of a single arbitrary sequence is used for low stringency PCR on a cRNA population a sample of the mRNA population is selected, primarily based on their match with the primer, rather than 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 RNA arbitrarily primed PCR (RAP-PCR) probes have an entirely different spectrum of abundances than the original mRNA population. The RAP-PCR 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.

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by random priming.

The ability of RAP probes to easily detect some rarer messages occurs because the probe complexity is reduced, meaning that some mRNAs are not sampled efficiently, and abundances are reproducibly distorted. Thus, obtaining nearly full coverage requires more than one RAP-PCR probe to be used. Nevertheless, using such probes is of utility because they reveal changes in RNA transcript abundance among the rarest transcripts, which are likely to include transcripts for some important regulatory proteins.

Deirdre R. Meldrum
University of Washington

Automated Minimum Residual Disease Quantifications

Deirdre R. Meldrum[1], Mark Holl[1], Tobias Mann[1], Charles Fisher[1], Mohan Saini[1], Daniel Sabath[1], David Cunningham[2], Stephen Moody[2], and William Pence[2]

[1]Genomation Laboratory, Department of Electrical Engineering, University of Washington, and [2]Orca Photonic Systems, Inc., Redmond, WA

The principle aim of residual disease analysis in patients with oncologic disorders is to gauge patient response to treatment and to enable early diagnosis of relapse. PCR is by far the most sensitive assay to detect residual disease and can enable a single cancer cell to be detected in a background of 105-106 normal cells. This is approximately 1000 times greater than the routine detection limit of cytological methods. After treatment, many patients have PCR-detectable tumor cells for prolonged periods of time without subsequently relapsing. Quantitative PCR techniques can distinguish between those PCR positive patients who have low or falling levels of cancer cells on sequential analysis from those who have levels that are increasing. This information may be used for early therapeutic intervention. Quantitative PCR techniques can quantify Minimal Residual Disease (MRD) in various forms of leukemias and can also be applied to the treatment of solid tumors. MRD analysis is focused on follicular lymphoma in the context of this project.

The needs of the MRD application, especially when it is ultimately extended in the clinical environment, result in unique requirements for automation. In addition to high throughput, MRD requires a high level of flexibility and programmed variability in sample processing. Existing commercial Realtime PCR instruments have been designed to target a broad market, rather than a specific application. The instrument being developed in this grant will optimize the optics to achieve high sensitivity and will completely automate the sample processing and analysis, allowing the required flexibility for MRD applications. By implementing statistical methods, the time-dependent kinetics data intrinsically contained in the Realtime PCR data stream is utilized to determine the initial copy number from an end-point measurement of DNA quantity. This amplification yield has not previously been measured.

In the R21 Phase I project, completed June 1, 2001, we developed and tested the proof-of-principle for a fluorescence analyzer research workstation for MRD quantification. We designed and built an optical analyzer test bed, ran experiments for optical train sensitivities and DNA detection sensitivities, and demonstrated robust, reliable, fast, and uniform, PCR thermal cycling. To estimate the sample initial copy count and the reaction yield, we developed and tested an estimation algorithm. In preparation for the R33 Phase II project, we completed a conceptual design of the MRD automated workstation and an MRD per sample analysis cost estimate.

In the R33 Phase II project, the R21 testbed and experiments will be extended to include a large population of realistic samples with sufficient numbers to support full statistical analysis of the results. To demonstrate automation feasibility and to support pre-clinical trials, the complete design, fabrication, and integration of a prototype Realtime PCR Workstation will be completed. This instrument will be based on the proof-of concepts validated in the R21 phase I. For high-throughput, automated analysis, the Realtime PCR Workstation will be integrated with an existing automated fluid handling system called Acapella-5K that was developed by the PI and her team (NIH NHGRI funding). We will develop an adaptive algorithm that converges toward an MRD estimate with a level of sensitivity and accuracy specified by the operator. For each patient DNA sample, multiple serial PCRs will be conducted to achieve the given levels of sensitivity and accuracy in the shortest time possible. We will initiate the design of a method for DNA extraction that is compatible with our automation methods for the Realtime PCR system. We will also analyze opportunities to extend use of the Realtime PCR instrument to other applications.

York Miller
Biochemical Mass Spectrometry Facility and University of Colorado Cancer Center, University of Colorado Health Sciences Center

Qualitative and Quantitative Proteomics Applied to the Identification of Cancer Biomarkers in Biological Fluids

Mark W. Duncan[1], Kim Fung[1], Joseph Zirrolli[1], Franco Basile[1], L. Michael Glode[2], and York Miller[3]

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Changes in both the nature and extent of protein expression are central to many pathologic processes. Characterizing these changes may lead to better diagnostic and prognostic markers, aid in our understanding of disease processes and assist with the discovery of improved therapeutic strategies. Over the last decade or so we have witnessed fundamental developments in mass spectrometry that now make it feasible to undertake high throughput, qualitative and quantitative assessment of expressed

proteins in biological tissues and fluids and consequently, these changes can now be characterized.

While methods based on protein identification following proteolytic digestion allow rapid protein identification, only a subset of the available data is used to make these assignments and important detail is often lost. There is considerable merit in examining gene products in greater detail, and our attention is now directed at absolute peptide and protein quantification and characterization of post translational modifications. Of particular interest to us in this regard is the nature and extent of oxidative damage to key proteins, as well as post transcriptional cleavages that produce biologically active peptides. To examine these issues we are developing a strategy based on both MALDI and electrospray mass spectrometry.

The direction and status of our proteomics endeavor will be presented, together with results from our detailed analysis of two biological fluids: human tear film, lung tissue and semen. Our methods are being applied to systematic search for protein biomarkers of specific cancers, and current attention is being directed towards the identification of both lung and prostate cancer.

Samuel C. Mok
Dana-Farber Harvard Cancer Center, Harvard Medical School

Identification of Ovarian Cancer Biomarkers by ProteinChip Array and SELDI/Mass Spectroscopy

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The objective of this study is to identify potential biomarkers in serum and plasma using the technique of Surface Enhanced Laser Desorption-Ionization (SELDI)-Mass Spectroscopy (MS) for early detection of ovarian cancer. Five different types of surface specific ProteinChip arrays were applied to screen for potential protein markers of molecular weight less than 100 kDa. A total of 74 age-matched serum samples (24 cases and 50 controls) and 33 age-matched plasma samples (22 cases and 11 controls) were used. Comparing mass spectra profile generated from these samples, several protein peaks were identified that consistently appeared in cases, but not in the controls. One protein peak (11723 Da) appeared in all subtypes of ovarian cancer cases. This 11723 Da protein was then further purified by affinity chromatography. Protein sequence data showed chain of haptoglobin. Haptoglobin is a?that this protein belongs to the tetramer generally secreted by the liver and involved in binding free

hemoglobin and preventing loss of iron from the body. Haptoglobin also has peroxidase activity when it is associated with hemoglobin. Intact haptoglobin levels have been shown to be elevated in ovarian cancer patients, but this is the first subunit may have greater sensitivity for ovarian cancer?evidence that the detection. More quantitative studies are underway.

Eric E. Monson
University of Michigan
Dynamic-Nanoplatforms

Eric E. Monson Dept of Chemistry, University of Michigan

In medical and biochemical research, when the domain of the Sample is reduced to micrometer regimes, e.g. living cells or their subcompartments, the real-time measurement of chemical and physical parameters with high spatial resolution and negligible perturbation of the sample becomes extremely challenging. A traditional strength of chemical sensors (optical, electrochemical, etc.) is the minimization of chemical interference between sensor and sample, achieved with the use of inert, "biofriendly" matrices or interfaces. However, when it comes to penetrating individual live cells, even the introduction of a submicron sensor tip can cause biological damage and resultant biochemical consequences. In contrast, individual molecular probes (free sensing dyes) are physically small enough but usually suffer from chemical interference between probe and cellular components.

Our recently developed PEBBLE sensors (Probes Encapsulated By Biologically Localized Embedding) are nanoscale spherical devices consisting of sensor molecules entrapped in a chemically inert matrix. This protective coating eliminates interferences such as protein binding and/or membrane/organelle sequestration which alter dye response. Conversely, the nanosensor matrix also provides protection to the cellular contents, enabling dyes that would usually be toxic to cells to be used for intracellular sensing. In addition, the inclusion of reference dyes allows quantitative, ratiometric fluorescence techniques to be used. PEBBLEs have been used to measure analytes such as calcium, potassium, nitric oxide, oxygen, chloride, sodium and glucose. Extensions of our existing PEBBLE technology are being developed as platforms for the early detection and treatment of cancer through our NCI funded UIP contract. In particular, targeted MRI, optical imaging, and photodynamic therapy will be supported by the same dynamic nano-platforms (DNP); nanoparticles with conserved cores. Other nano-particles have previously been employed, separately for MRI or for therapeutics, with varying degrees of success.

This project focuses on making and implementing conserved, multipurpose systems, using novel concepts of hybrid chemical materials. The DNP functions will include magnetic contrast, luminescence, multiple targeting and photodynamic therapy (PDT). The intent is for the DNP to provide direct massive delivery of reactive oxygen species (ROS) rather than release of PDT dyes. The practicality of the individual components of

this combinatory plan are supported by literature data and preliminary work in our own lab. Evaluation of photodynamic efficacy and image contrast enhancement will be examined in vivo and *in vitro* . The 9L intracranial gliosarcoma will serve as the model system for testing of DNP nanoparticles.

David Morris
University of Michigan

Use of Translation State Array Analysis (TSAA) to Identify Genes Translationally Regulated During the Cell Cycle

Kyle Serikawa [1], Roger Bumgarner[2], and David Morris[1] [1]Department of Biochemistry, and [2]Department of Microbiology, University of Washington, Seattle, WA

Translation state array analysis (TSAA) combines polysome fractionation by sucrose gradient centrifugation with DNA array technology, allowing a measure of the translational efficiency of individual mRNA molecules on a genome-wide scale. In order to test and extend this technology, we have applied it to an analysis of the cell cycle of Saccharomyces cerevisiae.

Cultures of a temperature sensitive cdc15-2 mutant strain were synchronized and sampled over time. Of ca. 6000 genes analyzed, approximately 10% seem to be translationally regulated during progression through the cell cycle. The TSAA results were confirmed for selected genes by Northern analysis of polysome fractions. Several cyclic patterns of change were observed with time. The translation states of families of genes involved in DNA replication, cell polarity and ribosome synthesis were found to be temporally coordinated.

In addition to characterizing translation state, TSAA also estimates transcript levels. The transcript patterns derived from TSAA were compared with published results generated by conventional DNA array expression analysis. The two sets of data were found to be in general agreement, even though the measurements were made at different times, in different laboratories and with different strains of S. cerevisiae.

Seajin Oh
SRI International

AFM-Based Technique Analyzes Expression in Cancer Cells

Seajin Oh, William Wright[1], Keith Laderoute([1]), and Eric R. Henderson[2] [1]SRI International, and [2]BioForce Laboratory, Inc.

We are developing an AFM-based technology involving a submicron scale probe array and, by using the array, directly analyzing the constituents of cells suspected or known to be involved in oncogenesis. In the method, AFM tips transfer protein probe molecules from reservoirs to a support, and deposit them at selected array positions. The process is repeated to complete a protein array. Target proteins are applied to the array, and the

binding events on the array molecules are monitored by AFM topography measurement. During this first year we prepared individual HIV gp120 molecule domains within each well in a 10 x 10 array, and detected binding events with HIV antibody molecules at the molecular level by AFM topography measurement. During the next period we plan to use a SAPK molecule and its probe molecule to demonstrate the applicability of the method to study the cells related to oncogenesis. In conjunction with the individual molecule binding event detection, we are also developing an electrostatic method to manipulate individual protein molecules. The method is based on the hypothesis that biological molecules are charged, allowing them to be attracted to, retained, and repelled from an AFM tip according to the electric polarity applied to the tip. Progress and challenges of the efforts will be presented.

Maria Pallavicini
University of California, San Francisco

Reliable Protein Identification from Stained or Unstained 2D gels using High Throughput MALDI TOF/TOF MS

Maria Pallavicini[1], Lan Huang[1], Katherine E. Williams[1], Carina Sihlbom1, Katalin F. Medzhiradszky[1], Jennifer M. Campbell[2], Peter Juhasz[2], Steven A. Martin[2], Marvin L. Vestal[2], Michael A. Baldwin[1], and A. L. Burlingame[1] [1]University of California, San Francisco, CA, and [2]Applied Biosystems, Framingham, MA

Mass spectrometry has become an essential tool for global deciphering of the human proteome. However, effective strategies to for comprehensive identification of proteins from cultured cells, tissues or other biological samples remain a major challenge. In particular, small samples of primary tumor tissues place severe limits on cells available for analyses. High throughput and highly sensitive methods are needed for protein identification on a global scale. Using high sensitivity tandem MS, i.e. MALDI-TOF/TOF or nanoflow HPLC-QqoaTOF, we are exploring methods for routine sequencing and identification of peptides below the threshold for silver or fluorescent gel staining for 1-D and 2-D SDS PAGE separations. Gel pixeling is an approach that is based upon arraying 1-D or 2-D gels into regions, regardless of protein staining, followed by mass spectrometric analyses of each gel region. Complete pixelation of unstained gels may be advantageous to for identification of proteins in low abundance.

We evaluated the gel pixeling approach for protein identification by digestion and analysis of protein spots from a stained gel prepared from lysates 5x106 NB4 acute promyelocytic cells with mass spectrometric analyses of unstained gel regions arrayed into pixels. Each pixel was analyzed using MALDI-TOF. 100 pixels were cut from a representative 2 cm square of a 2-D gel and visible spots were cut from the corresponding region of a 2nd gel stained with silver. All gel pieces were digested with trypsin and analyzed by MALDI-TOF to identify peptide masses for MS/MS. Subsequent high-energy MALDI-TOF/TOF CID spectra were recorded on selected ions from the same sample deposits. Further aliquots of selected samples were analyzed by nano-HPLC/ESI Pulsar. Tandem spectra were submitted to UCSF's MS-Tag or sequenced

manually. MS imaging of peptides in the pixelated region of an unstained gel identified 9 spots, only 5 of which were equivalent to silver-stained spots in a second gel. The remaining 4 yielded TOF/TOF data of sufficient quality for identification. Thus, the gel pixeling approach and mass spectrometry identified proteins below the level of detection with silver staining (Sypro ruby gave staining detection similar to silver). These preliminary results using the MALDI TOF/TOF mass spectrometer established its ability to generate high-energy CID spectra with low fmol sensitivity with sufficient speed to cope with such a strategy. Furthermore, most spectra were of sufficient quality to be suitable for de novo peptide sequencing, essential for dealing with unknown proteins or peptide sequences inconsistent with databases due to genomic sequence errors, differences in DNA splicing, chemical modifications or unpredicted enzyme cleavages. Although nano-HPLC/QSTAR analysis revealed additional peptides on analogous aliquots and provided high quality CID spectra, it was inherently slower.

In summary, these data support the concept of entire 1- or 2-D gel pixelation to provide improved dynamic range and comprehensive sequence information from limited amounts of material. Practical realization of such a strategy will require integration of sample handling robotics as well as further automation of the instrument operation, data acquisition and database searching to handle the large numbers of samples generated by gel pixelation. Future directions include implementation of full 1-D and 2-D gel arraying capabilities and application of this approach to identify protein signatures of acute promyelocytic leukemias that fail to respond to therapy. This work was supported by NCI CA86135 and NIH NCRR RR14606-01.

Mark W. Perlin
Cybergenetics

Automated Multiplex Genetic Analysis Technology

Mark W. Perlin and Alexander Sinelnikov Cybergenetics, Pittsburgh, PA

We have implemented automated dye separation methods. These have been incorporated into the TrueAllele software for use with capillary electrophoresis data, with extensive testing on ABI/310 CE data. The methods have also been adapted for use on gel electrophoresis systems, with testing on Hitachi FM/BIO2 data. The results generally outperform manual calibration of dye spectra on such instruments, and suggest that this time consuming, expensive, and error prone endeavor should be replaced by mathematical software.

We have developed new sizing and tracking algorithms. For 1D CE data, these sizing algorithms establish the boundary, and "zip up" the expected sizes and observed peaks. Use of backtracking and least square minimization methods help ensure robustness. TrueAllele now uses such methods for both size-based (e.g., ABI, BioVentures) and height-based (e.g., Promega) sizing standards. For 2D gel data, we have expectation-based lane tracking algorithms that proceed by row down the gel. These algorithms model the geometry of each band and row of size standard peaks, propagating results

from one row to the next. Extensive successful testing on a suite of twenty diverse gels suggests that this approach is quite robust, even with suboptimal data.

Our novel use of allelic ladders in genetic analysis appears to greatly improve the binning accuracy. We have developed such ladder-based methods for use with ordinary CA-repeat genetic data by pooling samples, and using this pool as a cross-gel standard. TrueAllele is then able to use this inexpensive ladder for allele assignment. We have tested this fragment identification approach on over 48,000 microsatellite genotypes as part of an automated genetic linkage study, achieving a discrepancy rate of 0.48% on the 99% of scorable genotypes.

We incorporated a TrueAllele capillary module for operation on high-throughput CE data. Supported platforms now include the ABI/310, ABI/3100, ABI/3700, and Molecular Dynamics MegaBACE 1000. The SpectruMedix SCE/9610 and other instruments will be included before the end of the first project year. The operation inputs the raw data files, automatically sets up the study, performs automated signal and allelic processing, and then outputs the results. User interaction is limited to two quality assurance steps, for post-signal and post-allelic review.

Automated high-throughput gene expression system for differential display analysis.

We automatically analyzed a differential display experiment, using data provided by a collaborator. This was done by horizontal and vertical transformation of an electrophoretic trace to superimpose its major features onto a reference trace. Then, the signals were subtracted, obtaining the differential pattern of relative peaks, binned by size. This result demonstrates the feasibility of fully automated differential display analysis.

Automated high-throughput biomarker system for chromosomal analysis.

TrueAllele has been optimized for automated allele calling and quality assessment. This year, we incorporated about twenty new quality assessment rules that identify particular deficiencies with the STR data. These deficiencies are related to specific laboratory data generation processes, and the use of our new statistical analyses and user interfaces can help diagnose quality issues.

By identifying the problematic data (and potential underlying causes), the user can focus on just the problematic data. There is no need to review the 90% of good data, which has high quality scores and no rule firings. This computer-based prioritization can eliminate most of the human time expended in the data review.

Daniel Pinkel
University of California, San Francisco

Technical Approaches for Efficient, High Precision Nucleic Acid Analysis using DNA Microarrays

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The biological utility of microarray analysis depends on several important factors including measurement precision, the amount of specimen material required for analysis, and cost. We have developed instrumentation and techniques for array production and analysis that offer improvements in all of these areas compared to current approaches. Our printer uses capillary tubes as printing pins, which allow printing from high density microtiter plate formats. The capillary pins use printing solutions much more frugally than quill pins, and their small tip diameters allow printing closely spaced spots. The close spacing reduces the size of the array and thus the amount of specimen material required for analysis. Arrays for genomic DNA copy number analysis are routinely m centers.?printed from 864 well plates with spots on 130.

Arrays are imaged using a custom built CCD camera-based system using mercury arc illumination. Images are chromatically corrected, flat, and distortion free. Specially designed excitation and emission filters allow acquisition of properly registered images from 4 fluorochromes with less then 0.1% cross talk. Two specimens and one reference can be analyzed on each array, thus doubling the amount of data per array.

Ratio standard deviations are substantially below 10% for analysis of DNA copy number variations in mammalian genomes (array CGH) using array elements derived from BAC clones. Ratios are quantitatively linear. Thus deletions and duplications affecting single clones can be reliably detected and amplification levels accurately quantified.

Indra Poola
Howard University College of Medicine
Molecular assay for estrogen receptors in breast tumors

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Breast tumors are currently diagnosed for the presence of classical estrogen receptor (ER") by immunohistochemistry to predict prognosis and select patients for anti-estrogen therapy. Based on the presence of ER", the response rate for the most widely used anti-estrogen, Tamoxifen, therapy is about 50%. Although the resistance/response to Tamoxifen is considered multifactorial, the most logical factors appear to be ER isoforms. A number of ER isoforms have been detected in breast tumors in addition to ER" that have distinct ligand binding and transcriptional properties. It strongly follows that response to a particular anti-estrogen therapy depends upon relative amounts of various ER isoforms in the tumor tissues. To derive a correlation between the composition of ER isoforms and response to a particular anti-estrogen therapy, highly sensitive methods are needed that can detect and quantify every isoform in a small

amount of tumor tissue. The problem with the currently used immunohistochemistry is that it is not highly sensitive, not quantitative, requires large amounts of tumor tissue to assay all known isoforms, expensive, and most importantly cannot distinguish between various ER isoforms. These problems are heightened in recent years since smaller smaller tumors are detected. In these cases, detection of even one isoform cannot be performed. The quantitation of ER isoform mRNAs is a more realistic approach to accomplish the evaluation of all ER isoforms than ER proteins in tumor samples. It follows that response to a particular anti-estrogen therapy depends upon relative amounts of various ER isoforms in the tumor tissues. To derive a correlation between the composition of ER isoforms and response to a particular anti-estrogen therapy, highly sensitive methods are needed that can detect and quantify every isoform in a small amount of tumor tissue.

Our project is to develop highly sensitive, clinically applicable molecular methods that can detect and quantify mRNAs of every ER isoform by Real-Time PCR approach. Our objectives are also to apply the developed methods and derive a correlation between the composition of various ER isoforms and response to a particular ligand using breast cancer cell lines and finally apply the developed assays to identify patients who are most likely to respond to a particular anti-estrogen therapy based on the ER isoform mRNA profiles. to assay all known isoforms, expensive, and most importantly cannot distinguish between various ER isoforms. These problems are heightened in recent years since smaller smaller tumors are detected. In these cases, detection of even one isoform cannot be performed. The quantitation of ER isoform mRNAs is a more realistic approach to accomplish the evaluation of all ER isoforms than ER proteins in tumor samples.

Thus far we have developed assay for the detection and quantification of ER" wild type mRNA copy numbers in breast tumor samples. Our results show that as low as 0.5 ng of total RNA from breast cancer cell lines and 4 ng of total RNA from tumor samples is sufficient to detect and quantify Wild type ER".We have begun developing methods for other ER isoforms. Each isoform mRNA quantitation is achieved in comparison to a standard cRNA that is prepared for that particular isoform and specific primers and probes profiles.

Glenn D. Prestwich
Echelon Research Laboratories Inc.

Diagnosis of Cancer by Detection of Phosphoinositide 3-Kinase and its Lipid Product, PtdIns(3,4,5)P3

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Phosphoinositide polyphosphates (PtdInsPns) are biosynthesized by lipid kinases and phosphatases and are key lipid second messengers in cellular signaling. Phosphatidylinositol 3-kinase (PI 3-K) has been identified in regulation of cellular processes such as protein trafficking, proliferation and differentiation, tumorigenesis, and apoptosis. PI 3-K and its lipid product PtdIns(3,4,5)P3 are potential tumor markers for the early detection of cancer. Echelon Research Laboratories Inc. has now developed reagents and assay platforms to test the hypothesis that high PI 3-K activity and overabundance of the lipid product PtdIns(3,4,5)P3 are correlated with the appearance or progression of metastatic disease. First, we have developed and produced two monoclonal antibodies to PtdIns(3,4,5)P3, one suited for intracellular localization and one optimized for detection of the purified lipid in extracts. Second, we have produced prototypes of two non-radioactive microtiter plate assay platforms for detection of PtdIns(3,4,5)P3 produced by PI 3-K acting on PtdIns(4,5)P2. These platforms employ a recombinant PtdIns(3,4,5)P3-binding protein that is readily produced and purified with a bacterial expression system. Both assays, a competitive ELISA and a competitive fluorogenic assay, are formatted for conversion to high-throughput screening. Third, we have initiated experiments for immunodetection of PtdIns(3,4,5)P3 in pathology samples in clinical materials from breast, ovarian, prostate, and colorectal cancers using specimens from the Cooperative Human Tissue Network. These data will test the hypothesis that these lipid markers have diagnostic and predictive value.

J.M. Ramsey
Oak Ridge National Laboratory

Microfabricated Fluidic Devices for Protein and Peptide Mapping

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Miniaturized chemical instruments, Lab-on-a-Chip technologies, are being developed for rapid, comprehensive analysis of cellular proteins, as an alternative to the slow and labor-intensive 2D gel methods currently used for protein mapping. The microfabricated devices integrate on a single structure, elements that enable multidimensional separations of protein or peptide mixtures and on-chip labeling for fluorescence detection of rapidly migrating analyte bands. Moreover, the longer-term goal is to incorporate chemical and biochemical processing procedures and electrospray capability into the microfabricated devices. It is anticipated that these devices will exhibit many advantages including small size, inexpensive fabrication, high speed, low volume materials consumption, high throughput, and automated operation. These attributes are also attractive for eventually addressing the ability to perform protein mapping studies on individual cells under high throughput conditions. 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. We have also demonstrated a microchip device that accomplishes field gradient focusing of proteins, a method for concentrating and separating proteins. Field

gradient focusing is similar to isoelectric focusing but concentrates materials in a charged state rather than at their isoelectric point, thus reducing precipitation problems. Proteins and peptides have been concentrated nearly 1000 times by this process while also providing modest chemical separation. We are exploring the use of field gradient focusing as a front-end processor for feeding the two-dimensional separation devices discussed above. The status of our efforts to demonstrate an integrated protein processor will be provided.

William N. Rom
NYU School of Medicine

Lung Specific Expression of Dominant Negative Mutant p53 in Transgenic Mice Increases Spontaneous and Benzopyrene Induced Lung Cancer

Kam Meng Tchou-Wong Ph.D. and William N. Rom M.D. NYU School of Medicine

Mutations in the p53 gene have been implicated to play an important role in the development of various human cancers. To evaluate the importance of p53 in lung cancer formation in vivo, a transgenic mouse model was established by utilizing an 898 base pair mouse Clara cell specific (mCC10) promoter to target the lung specific expression of a dominant negative mutant form of p53 (dnp53a). In transgenic mice, the dnp53 protein was expressed in two transgenic founder lines exclusively in the lungs, while its expression was absent in the brain, heart, intestine, kidney, muscle, spleen, and uterus. The spontaneous incidence of lung cancer in average 18-month old transgenic mice was 45%(14/31) whereas age-matched control mice was 20%(4/20) (OR 3.3,CI 0.8-1.6.3,p=0.079). In addition to the increased spontaneous tumor incidence, these mice were more susceptible to the development of lung adenocarcimnoma following intratracheal instillation of benzo(a)pyrene. Six months after instillation of benzo(a)pyrene, the tumor incidence in the wild type and transgenic mice was 39% and 73% respectively. Thus, our data suggests that p53 function is crucial for protecting mice from spontaneous and benzo(a)pyrene-induced lung cancers.

Stanley Shackney
Allegheny General Hospital and the University of Pittsburgh

Laser scanning cytometry (LSC): A technology for performing multiple cell-based measurements per sample in groups of five correlated measurements per cell

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Laser scanning cytometry is a relatively new technology for performing multiple correlated fluorescence measurements per cell on relatively large number of cells. We have developed techniques for performing five correlated fluorescence measurements per cell in human epithelial cells. Human tumor samples that are disaggregated into single cell suspensions often contain 1-4 million cells, which can be fixed and deposited

on slides in aliquots of 50,000 cells per slide. The cells on each slide can be stained with five fluorescent probes. After cell losses during slide preparation are accounted for, and after data from overlapping cells and cell clumps are removed, multiple correlated cell-based data on 5,000-10,000 cells are obtained.

Quantitative intracellular levels of multiple cell constituents can be measured in human tumors, and compared with normal values. Clustering patterns of up to five intracellular molecular abnormalities can be identified within individual cells. Multiple five color panels can be performed on each tumor, making it possible to establish extensive profiles of clustered intracellular abnormalities in human tumors that may be of prognostic and diagnostic value.

Michael Sheldon
Texas Children's Cancer Center, Baylor College of Medicine

Molecular Classification of Osteosarcoma

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Osteosarcoma is the most common malignant bone tumor in children. Approximately 80% of patients present with non-metastatic disease. After the diagnosis is made by a biopsy, treatment involves 3-4 courses of neoadjuvant chemotherapy before definitive surgery, followed by post-operative chemotherapy. With currently available treatment, approximately 30-40% of patients with non-metastatic disease relapse after therapy. There is no prognostic factor that can be used at the time of diagnosis to predict which patients will have a high risk of relapse. The only significant prognostic factor predicting the outcome in a patient with non-metastatic osteosarcoma is the histopathologic response of the primary tumor resected at the time of definitive surgery. The degree of necrosis in the primary tumor is a reflection of the tumor response to neoadjuvant chemotherapy. Higher degree of necrosis is associated with lower risk of relapse and therefore better outcome. Patients with lower degree of necrosis have a much higher risk of relapse and poor outcome even after complete resection of the primary tumor.

Unfortunately this poor outcome cannot be altered despite modification of post-operative chemotherapy to account for the resistance of the primary tumor to neoadjuvant chemotherapy. Thus there is an urgent need to identify prognostic factors that can be used at the time of diagnosis to recognize the subtypes of osteosarcoma patients that have high risk of relapse so that more appropriate chemotherapy can be used at the outset to improve the outcome. We propose to establish a molecular classification system to distinguish such subsets of osteosarcomas based on their gene expression profiles. Using cDNA microarrays, we will investigate the gene expression

profiles of the tumor tissues at the time of biopsy and definitive surgery. These profiles will be correlated with clinical outcome. In addition, we also plan to compare the gene expression profiles of the primary tumor and those of the metastatic lesions. The goals of this project include (1) the validation and optimization of cDNA microarray technology for gene expression profiling of clinical specimens and (2) establishing the relevant gene expression profiles for molecular classification of osteosarcoma by correlating these profiles with clinical outcome, chemosensitivity, and metastatic potential. So far we have optimized the T7 linear amplification of RNA for analyzing small quantities of specimens such as biopsies and microdissected materials. With one round of T7 amplification, we can now routinely obtain 700-800 fold amplification from 100 ng of total RNA as starting materials and with minimal distortion of the expression profiles.

A. Dean Sherry
University of Texas at Dallas

New Functional MRI Agents Based upon Magnetic Transfer

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Current Gd3+-based MRI agents rely upon rapid exchange of a metal ion-bound water molecule with bulk water. We recently demonstrated that complexes with extremely slow bound water exchange rates may provide advantages in the design of biologically responsive agents. Several new lanthanide-ligand systems have now been synthesized and examined by high resolution NMR. It was found that the metal ion-bound water lifetimes can be modulated over a wide range (from nano- to milli-seconds) by modifying the groups attached to the extended side-chain amide groups of the ligand and by judicial choice of the lanthanide ion. s to 1.2 ms show a separate?M298) of 100 ?Complexes with bound water lifetimes (bound water signal in their 1H or 17O NMR spectra and pre-saturation of those bound water signals results in 50 80% decrease in intensity of the bulk water resonance. This forms the basis of a new class of MRI contrast agents called MT contrast agents. As exchange between Ln3+-bound and bulk water protons can be modulated by pH, these new MT contrast agents can be used as a pH indicator to report the pH values directly through the intensity of MR images. We have demonstrated the effectiveness of such agents in MR phantom experiments and are currently evaluating possible toxicity of these agents in animals.

Michael Showe
The Wistar Institute

Characterization of Cutaneous T-cell Lymphoma by RNA expression profiling

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Cutaneous T-cell Lymphoma (CTCL) is an orphan disease for which there are many treatments but no cure. It affects 15-20,000 patients in the United States. Stages of increasing severity range from low level skin involvement to skin tumors (Mycosis Fungoides), to further involvement of peripheral organs such as lymph nodes and lungs and to a leukemic form, Sezary Syndrome (SS), with circulating malignant cells. Progression from stage to stage may occur over many years providing numerous opportunities for RNA expression characterization and possibilities for clinical intervention.

We have been using cDNA microarrays to develop gene expression profiles that are representative of different stages of CTCL starting with samples from patients with Sezary Syndrome (SS) the leukemic form. Approximately 4500 probes were prepared from the Research Genetics 200 and 201 series of clones by selecting plates which included known genes. These probes were spotted on 25 x 75 mm nylon filters with a GM 417 arrayer using 300 micron pins at 750 micron spacing, depositing approximately 2 nl of DNA solution at 0.1 mg/ml . Target total RNA was amplified as described by Eberwine et al. and labeled with 33-CTP. After hybridization, an average of 75% of the spots had signals greater than 2-fold over background (60%-95%). Statistical analysis of 2,200 clones on 20 sets of duplicate microarrays shows error in expression determination decreases from +/- 50% for the lowest expressed genes to +/- 23% for the highest over a dynamic range of 3 orders of magnitude.

We have examined gene expression profiles of Tcells from 29 SS patients and 9 controls. The controls include normal PBL activated with PHA and T-cells skewed to Th-1 or Th-2 type by culturing with appropriate cytokine and anti-cytokine combinations. Both univariate and hierarchical clustering statistics were applied to the data. Patient and control groups were clearly distinguished from each other, and the patients appear to form at least three groups distinguished from each other by groups of differentially expressed genes. Patients express over two hundred genes at a statistically different level from the controls (P=0.001), and permutation of patients and controls suggests that no more than 50 of these can be due to chance.

Branimir I. Sikic
Stanford University School of Medicine

Genomic Profiling of Ovarian Cancers

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Ovarian cancer is the fifth leading cause of cancer deaths in women, and the major cause of death from cancers of the reproductive tract. We have used complementary DNA (cDNA) microarrays consisting of approximately 23,000 known genes and ESTs to examine gene expression patterns in 42 ovarian tumor specimens collected at Stanford over the past 20 years, as well as 17 ovarian cell lines. The comparison between individual tumor and tumor-derived cell lines has revealed variation in gene expression patterns that reflect histologic and physiologic variation within the tumor samples. Similarly, the comparison of the ovarian patterns to patterns identified in other tumor types suggests that subsets of genes are relatively ovarian-carcinoma specific, and may therefore distinguish aspects of ovarian cancer cell biology. A set of genes has been identified that distinguish a particularly virulent subset of ovarian cancers, clear cell carcinomas, from other subtypes. Ongoing aspects of this proposal are: (1) Additional accrual of ovarian cancers from Stanford, as well as via collaborations with the University of Pennsylvania (Steven Johnson and Peter O'Dwyer) and the Norwegian Radium Institute (Anne-Lise Borresen-Dale), to increase the statistical power of our analyses. (2) Evaluation of the diagnostic value of specific genes and gene clusters to distinguish ovarian epithelial cancers from other cancer types, and to differentiate among histopathological grades and subtypes of ovarian cancers. (3) Identification of genes which act as determinants for responsiveness to chemotherapies for ovarian cancers. (4) Identification of genes which are prognostic for patient survival.

Note: Dr. Sikic's IMAT award began in April, 2001, entitled “Genomic Profiling of Unknown Primary Cancers,” R33 CA 89830.

The aims of this project are as follows: 1) Determination of the diagnostic cluster of gene expression for known tumor types in the Stanford Microarray Database, and acquisition of other tumor types as needed to characterize the diagnostic possibilities for unknown primary cancers (UPCs). (2) Acquisition, gene expression profiling, and diagnostic classification of unknown primary cancer specimens. This aim involves a collaboration with the world's leading center for the clinical evaluation of unknown primary cancers, the Sarah Cannon Cancer Center in Nashville. (3) Evaluation of a panel of histospecific antisera for diagnostic utility with UPC specimens. This aim will utilize retrospective archived specimens as well as prospectively acquired samples from this project. (4) Identification of specific or clustered gene expression associated with response to therapies and survival of patients with UPC.

Robert H. Singer
Albert Einstein College of Medicine

FISH & Chips: Single Cell Analysis of Cancer Gene Expression

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We aim to study transcription of genes known to be involved in carcinogenesis to further

the molecular classification of cancer and build the basis for a medical diagnostic tool. To this end, our laboratory has developed a high-resolution Fluorescence *in situ* Hybridization (FISH) technique that has been proven to discern single molecules of mRNA. We have achieved the simultaneous detection of ten transcript species using multiplexed FISH with combinations of four fluorophores encoding the gene identities (spectral barcodes). The fluorescence images are analyzed by computer software that detects the sites of transcription for each gene by locating the points of maximum intensity and interpreting the signal combinations. Single cell analysis is performed by processing an image of a nuclear counter-stain using a segmentation algorithm. This allows the direct observation of correlated transcriptional events and their organization in an intact interphase nucleus.

This technique represents a means by which gene expression patterns can be studied in a novel way on a single cell basis. It allows a three-dimensional, structural representation of the vast transcriptional data obtained from microarrays. Genes identified in microarray studies can be analyzed for finely demarcated expression *in situ* , thereby preserving tissue and cellular architecture. The development of our technology is based upon automation of every step of transcriptional profiling, from gene selection using microarray databases accessible via the Internet to image acquisition using robotics and computer-based analysis of the assay results. Applying FISH & Chips to different cell types or cultures under different conditions will lead to the accumulation of single-cell transcriptional profiles that we believe will give unique insight into cell phenotype and physiology.

Two considerations make this approach to studying cancer gene expression ideal. First, cancer, by its clonal nature, is the derangement of a very small population of cells. Second, oncogenesis is marked by aberrantly over-expressed and under-expressed genes. FISH & Chips provides the ability to assay simultaneously a large number of critical tumor-suppressors and oncogenes with a cell-population approach. This technology has been proven to be molecularly sensitive, theoretically allowing for the visualization of rare transcripts. Tissue specimens obtained for histological evaluation may contain normal as well as pathological cell populations. The assay will provide for fine examination of gene activity in a way that would be suited for use in histopathology. The end result of this development is the use of a database of transcription profiles as a guide for the classification of unknown clinical samples for assisted diagnosis, prognosis and selection of treatment.

Martin Stanton
Nobuko Hamaguchi and Martin Stanton: Rosenstiel Basic Medical Sciences
Protein Detection using Aptamer Beacons

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We have developed a new class of nucleic acid sensor molecules, which we term Aptamer Beacons, for detecting proteins and other ligands. Aptamer beacons can adopt 2 or more conformations, one of which allows ligand binding. A fluorescence-quenching pair is used to report changes in conformation induced by ligand binding. To demonstrate this method, we engineered two previously selected aptamers, an anti-thrombin aptamer and an anti-Taq polymerase aptamer, into aptamer beacons. The thrombin aptamer beacon was engineered by adding nucleotides to the 5-end that are complementary to nucleotides at the 3-end of the aptamer. In the absence of thrombin, the added nucleotides will form a duplex with the 3-end, forcing the aptamer beacon into a stem-loop structure. In the presence of thrombin, the aptamer beacon forms the ligand-binding g-quartet structure.

This conformational change causes a change in the distance between a fluorophore attached to the 5-end and a quencher attached to the 3-end, leading to a detectable optical signal. Similar methods were used to engineer the Taq polymerase aptamer beacon. Aptamer beacon seem to provide a general and sensitive tool for detecting proteins and other chemical compounds.

William M. Strauss
Harvard Institute of Human Genetics
PNA interference mapping: a new technique for RNA structural analysis

William M. Strauss Harvard Institute of Human Genetics, Harvard Medical

We describe a new technology, PNA interference mapping (P-IMP), for the structural analysis of expressed RNA sequences in living cells. Using the non-coding RNA Xist, as a model system, we demonstrate that a specific region of the Xist RNA mediates binding to the inactive X-chromosome. The chromatin-binding region of Xist was mapped using P-IMP. In the reported experiments a single 19-bp antisense cell-permeating PNA targeted against a particular region of Xist RNA caused the disruption of a macromolecular-nuclear structure, the inactive X-chromosome. The association of the inactive X-chromosome with macro histone H2a is also disturbed by P-IMP. Neither scramble, mismatch, sense nor PNA directed at other regions of Xist have any effect on Xist coating, or the inactive X-chromosome. Moreover, PNA-conjugates have no effect on steady state levels of Xist, X-linked, or autosomal RNAs. These data demonstrate that the P-IMP is specific, and suggests that P-IMP is a true interference technology. Thus P-IMP may be a rapid method to determine the critical determinants in effector RNAs. These data suggest an important experimental paradigm for connecting nucleic acid sequence information to functional consequences in living cells.

John C. Sutherland
Brookhaven National Laboratory
DNA Damage Quantitation by Single Molecule Laser Sizing

John C. Sutherland, Elena M. Filippova, John G. Trunk, Denise C. Monteleone, and Stephen R. Quake Biology Department, Brookhaven National Laboratory, Upton, NY 11973 & Department of Applied Physics, Institute of Technology

We are developing a method to determine the length (in base-pairs) of double-stranded fluorochrome-labeled individual DNA molecules passing through a narrow microfabricated channel using laser-induced fluorescence. From these data, we compute the number-average length of the population of DNA molecules in the sample. Comparing the number average molecular lengths of populations treated with carcinogens such as ionizing radiation and various combinations of damage-specific repair enzymes with the average molecular length of untreated controls yields quantitative measures of the frequency of double strand breaks and several classes of clustered damages. This technology will:

- improve the sensitivity (lesions per unit length of DNA) and
- reduce the time required to quantify clustered lesions while
- reducing the quantity of DNA required for an assay perhaps to the level of the DNA in a single mammalian cell.

We have completed construction of a prototype single-molecule sizing instrument and demonstrated the feasibility of the approach by determining the number average length of restriction fragments of the 30 kbp DNA of bacteriophage T7, obtaining values close to those calculated from theory. Typical data showing fluorescence over a period of 20 s are shown in Figure 1. Future thrusts involve increasing the lengths of the DNAs studied, thereby increasing the sensitivity limits of the damage levels measured.

This technology should allow quantification of the very low levels of damage characteristic of environmental and routine medical exposures. It can be applied to DNA damaged *in situ* , thus facilitating basic research on DNA damage and repair and the relationship of these processes to mutation induction and carcinogenesis. Potential clinical applications include rapid determination of the ability of normal and tumor cells to repair damage, thus permitting identification of individuals who may be at elevated risk or the optimum agents to use against a particular cancer.

James A. Swenberg
University of North Carolina, Chapel Hill

Ultrasensitive Methods for Human DNA Adduct Quantitation

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There is great interest in expanding the use of biomarkers to understand basic mechanisms of carcinogenesis, measure occupational and environmental exposures to chemicals and to determine endogenous sources of DNA damage. The primary focus of

our research is the development of ultrasensitive techniques for monitoring DNA adduct formation using liquid chromatography/electrospray tandem mass spectroscopy (LC-MS/MS).

We have developed LC-MS/MS methods for the analysis of dAdo) and 7-hydroxyethylguanine and have made?1,N6-ethenodeoxyadenosine (significant progress in the development of LC-MS/MS methods for the analysis of dGuo),?3,N4-ethenodeoxycytosine, N2,3-ethenodeoxyguanosine (N2,3- dGuo) and the exocyclic propano guanine adducts?1,N2-ethenodeoxyguanosine (1,N2- derived from crotonaldehyde (CdGuo-1 & -2). These adducts are formed in DNA from animals and humans exposed to vinyl chloride, simple alkylating agents and endogenous processes, as indicated in by their presence in DNA of unexposed dAdo assay?animals and humans. The limit of quantitation in DNA using the dAdo adducts/109 normal deoxyadenosine. This?developed was estimated to be 5 assay used immunoaffinity chromatography as a clean up method prior to LC-MS/MS analysis. The detection limit in DNA using the 7HEG method is ~5 adducts/108 normal guanine. The LC-MS/MS method developed for 7HEG was as sensitive as a previously developed GC-MS assay and eliminated two days of sample preparation and a contaminant in unexposed, endogenous samples.

Preliminary results with standards showed that LC-MS/MS analysis of dGuo gave a signal to noise ratio (S/N) of 30 when 1 fmol is injected.?N2,3- This technology coupled with immunoaffinity chromatography that results in 80-90 Gua from DNA samples suggests that this is a very feasible recovery of technology for the analysis of this adduct. In addition, preliminary results showed that we were able to detect 750 amol of the CdGuo-1 adduct with a signal-to-noise ratio of between 68-114, indicating the sensitivity of this adduct is great enough that LC-MS/MS is a promising technology for the analysis of this adduct. In the future we will continue the development of assays for the etheno adducts and the propano adducts derived from crotonaldehyde as well as begin the development for an assay for propano adducts derived from acrolein. In addition, we will develop aptamers to use in affinity chromatography for clean up of adducts prior to LC-MS/MS analysis and for use in slot blot methods for quantitation.

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Strategies for Functional Cloning, Sorting and Expression Profiling of Genes That May Be Relevant to Cancer

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A major challenge of the post sequencing era is to assign function to the estimated 30,000 genes that reside in the human genome. We have developed a modified functional expression cloning strategy to identify human genes that encode diverse DNA and RNA-binding proteins. Some of these proteins may be provide insight to our understanding of cancer by virtue of their ability to regulate gene expression or maintain the structural integrity of the genome. Over a hundred cDNAs have been isolated in our pilot screen and additional screens are planned from different libraries. We are also developing a sorting phase that, in the absence of subcloning and protein purification, can distinguish among different classes of nucleic acid binding proteins according to their encoded binding properties for particular types of nucleic acid substrates. The cellular transcriptional activity of genes identified in our screen, (as well as that of most genes encoded by the human genome), is currently being examined using Affymetrix GeneChip-based expression profiling. This analysis is being performed in the context of approximately 12,500 full-length human genes and 47,500 EST clusters with the goal of building a comprehensive database of transcription patterns for specific cancer cell lines and biopsies that represent a wide range of human malignancies. In addition to using existing methods, we are also developing new data mining tools to help identify select groups of genes whose expression profile distinguishes particular types of cancer. A new classification method will be described that is based on analysis of selectively expressed genes through their present versus absent calls and reduces reliance on scaling or normalization when comparing data across subjects.

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Using Gene and Proteomic Methods to Profile Cancer Cells

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Alternations in cell or tissue morphology. While the foundation of modern cancer diagnosis and treatment, this approach has obvious limitations. The most conspicuous example is those tumors that lack obvious phenotypic traits (the"undifferentiated tumor" problem). At the same time, recent advances in gene expression technology have made it possible to assess the activity of the entire repertoire of human genes within a tumor, or at least a few million cells therefrom. Though capable of providing phenotypic information leading to a molecular classification of cancer (as evidenced by the Director's Challenge Awards and numerous published papers and abstracts), the real value of this approach is to identify phenotypic traits that bespeak functional defects within tumors.

Though mass methods as currently employed in conventional DNA chip technologies have already proven capable of doing so, it is also obvious that cancer is a heterogeneous tissue, even if derived from a primordial clone of neoplastic cells. Continual regional clonal evolution of tumor cells suggests the ability to assess molecular phenotype in a single or few tumor cells would be an important method of developing more accurate profiles of cancer cells. Our goal in our studies of cooperative

group-derived childhood sarcomas, as well as institutional studies of breast cancer (both with limited starting material) is to develop amplification methods that will allow us to use limited starting material to develop such profiles, and compare them with overall molecular phenotypes using the data mining tools currently under development. We are also interested in assessing the potential impact of alternate molecular technologies on these profiles, particularly confirmatory methods focused on protein identification and characterization as well as rapid, quantitative, and accurate assessment of expression levels of limited numbers of genes important in certain biologic processes identified from our ongoing microarray studies.

Jan Trnovsky
One Cell Systems

Detection of Telomerase mRNA in Individual Leukemia Cells by FISH and Flow Cytometry

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Fluorescence *in situ* hybridization (FISH) assays are increasingly used to identify chromosomal translocations, deletions and other chromosomal abnormalities associated with specific cancers. However, current assay formats rely on manual and labor intensive microscopic examinations making rare cell detection impractical. For early diagnosis of leukemias and for detecting therapeutic relapse, high throughput assays which can detect one cancer cell in the presence of at least 1,000 non-cancerous cells are needed. Telomerase mRNA (tmRNA), is a potentially valuable marker for early cancer cell detection because carcinogenesis seems to be dependent on telomerase activation.

We have developed a FISH method for detection of tmRNA in individual encapsulated cells using a plurality of fluorescein labeled oligo probes. This method, combined with flow cytometry, allowed detection of 0.001% of a model cells (HL-60) spiked into normal human peripheral blood mononuclear cells (PBMCs). Signal to noise ratio of 4.45:1 was obtained in flow cytometry measurements. We analyzed five different human PBMCs, each comprised of a mixture of cells collected from three different individuals and found that the noise level from normal cells is below the noise level that would prevent rare cancer cell detection. Encapsulation was used to prevent cell loss, mainly the consequence of cell clumping which frequently occurs under harsh hybridization condition. Cancer cells were not differentially lost during the encapsulation process.

In the near future we will profile telomerase mRNA expression in peripheral blood samples from patients with leukemias and lymphomas. Additionally, gel microdrop (GMD)-based reference standards, loaded with pre-determined copies of tmRNA, will be developed for determination of tmRNA copy numbers in individual cancer cells.

Ching-Hsuan Tung
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Medical School
In vivo imaging of matrix metalloprotease

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The goal of the current research is to visualize the in vivo activity of Matrix Metalloproteinase-2 (MMP-2), an enzyme critically involved in angiogenesis, tumor progression and formation of metastases. A near-infrared fluorescence (NIRF) contrast agent was developed which was highly activatable by MMP-2 induced conversion. Signal characteristics of the probe were studied *in vitro* using pure enzymes. The MMP-2 sensitive probe was activated by MMP-2 showing an up to 850 % increase of the NIRF signal. This enzymatic activation can be blocked by MMP-2 inhibitors. Animal tumor models were established using a MMP-2 positive (human fibrosarcoma) and a MMP-2 negative tumor cell line (mammary adenocarcinoma). Both tumors were co-implanted into nude mice and optically imaged following IV administration of the MMP-2 sensitive probe. MMP-2 positive tumors were easily identified by a bright NIRF-signal as early as 1 hour after IV injection of the MMP-2 probe while contralateral MMP-2 negative tumors showed little NIRF-signal.

During the past year, we have shown for the first time the feasibility of imaging MMP-2 enzyme activity in vivo using new near-infrared optical imaging technology and novel MMP-2 sensitive probes. As the project move into phase II, we will further optimize the MMP probes to gain better amplification of signal and protease selectivity. The optimized probes will be then tested in animal models of cancer and during anti-MMP-2 drug treatment.

Timothy D. Veenstra
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Application of High Throughput Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Strategies for the Molecular Analysis of Cancer

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The rapid increase in the number of completed sequences, particularly the anticipated release of the complete human genome, has lead to the development of techniques to use this data for the analysis of biological systems. One such area being developed is proteomics the study of the entire protein complement of a cell, tissue, or organism, under a specific set of conditions. While methods to make global measurements of gene expression at the mRNA level have been developed, these methods do not provide direct measurements of protein abundance and several studies have shown that mRNA

and protein abundances may show poor correlation. Global analysis of gene expression at the protein level provides the most informative target about the specific functions of individual genes and their interactions in cellular pathways and networks. To be effective, proteomics must be high throughput and quantitative so relative differences in protein abundances can be determined for thousands of proteins in a short time period. Efforts in our lab have been directed to establishing high throughput proteomics methods that allow for multiple perturbations of a cell system to be analyzed in a short period of time.

To meet this goal we have pioneered the concept of the accurate mass tags (AMTs) that can be used as biomarkers to uniquely identify specific proteins within a proteome. AMTs are identified by correlating the partial peptide sequence information obtained using tandem mass spectrometry (MS/MS) using conventional mass spectrometry instrumentation with the high mass measurement accuracy (MMA) data achievable using Fourier transform ion cyclotron resonance mass spectrometry (FTICR). The generation of a database of AMTs to identify proteins from an organism enables high throughput studies designed to measure differences in the relative abundances of proteins from two proteome samples. We have applied this technology to identify AMTs for the mouse B16 melanoma cell line and have also established methods to quantify differences in the relative abundances of proteins in this cell line using stable isotope labeling. The future plans are to establish as many AMTs as possible for this cell line and use these biomarkers to identify changes in protein abundances between two different tumor cell variants (i.e., B16 vs. B16F10). The ultimate goal of this research is to develop this technology to enable the measurement of differences in protein abundances between primary and metastatic tumors.

Suzanne D. Vernon
Centers for Disease Control and Prevention
Molecular Signatures of Cervical Cancer: An EDRN Study

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The US National Cancer Institute's Early Detection Research Network (EDRN) is a multi-site multi-disciplinary collaboration to facilitate the transfer of basic science discoveries into clinical practice. Our project is directed toward early detection of cervical cancer. We are conducting a molecular epidemiologic study of preinvasive cervical lesions in a high-risk urban population. Identifying and implementing molecular markers for early detection of preinvasive cervical disease requires that sampling methods be optimized for disease representation and molecular preservation.

The epidemiologic and clinical data are collected from interview and chart-review. The cervical disease status is established from cytology, colposcopy, and biopsy results.

Biologic samples collected include exfoliated cervical cytology cells, cervical mucosal secretions and blood. The major technologic challenges are to develop optimal high through-put methods for collecting and archiving DNA, RNA and protein information from each sample. The stability of archival samples with linked epidemiologic data is of utmost importance for biomarker validation by consortiums such as EDRN.

To date we have focused on optimizing collection and nucleic acid extraction methods for the cervical cytology samples. The optimized method uses a detergent-based stabilibizing media and a simplified total nucleic acid extraction. These nucleic acids can be readily used in PCR and RT-PCR assays. We are developing methods for efficient evaluation of the quality of the sample for gene expression profiling and for ensuring long-term RNA stability. The approaches include gel electrophoresis, size-specific multiplex RT-PCR, real time RT-PCR and cDNA archiving.

The mucosal samples will be the source for proteins to evaluate local inflammatory and immune responses. We are exploring single-tube multi-analyte profiling to simultaneously perform multiple assays in limited material.

Marc Vidal
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Open-reading-frame sequence tags (OSTs) support the existence of at least 17,300 genes in C. elegans

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The genome sequences of Caenorhabditis elegans, Drosophila melanogaster and Arabidopsis thaliana have been predicted to contain 19,000, 13,600 and 25,500 genes, respectively1,2,3. Before this information can be fully used for evolutionary and functional studies, several issues need to be addressed. Firstly, the gene number estimates obtained in silico and not yet supported by any experimental data need to be verified. For example, it seems biologically paradoxical that C. elegans would have 50% more genes than D. melanogaster. Secondly, intron/exon predictions need to be experimentally tested. Thirdly, complete sets of open reading frames (ORFs), or ORFeomes4, need to be cloned into various expression vectors. To simultaneously address these issues, we have designed and applied to C. elegans the following

strategy. Predicted ORFs are PCR amplified from a highly representative cDNA library4 using ORF-specific primers, cloned by Gateway(tm) recombination cloning4-6, and then sequenced to generate ORF sequence tags, or "OSTs", as a way to verify identity and splicing. In a sample (n=1,222) of the nearly 10,000 genes predicted ab initio, i.e. for which no expressed sequence tag (EST) is available so far, at least 70% could be verified by OSTs. We also observed that 27% of these experimentally confirmed genes have a different structure from that predicted by GeneFinder. Most importantly, we now have experimental evidence that supports the existence of at least 17,300 genes in C. elegans. Hence we suggest that gene counts based primarily upon ESTs may underestimate the number of genes, in human and in other organisms.

Peter K. Vogt
The Scripps Research Institute
Inhibiting Myc-Max Dimerization

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We have explored the possibility of intervening in protein-protein interactions involving oncoproteins. In a high throughput test, based on fluorescence resonance energy transfer, we have screened for inhibitors of Myc/Max dimerization, isolating candidate molecules. These can be shown by independent immuno assays to interfere with Myc/Max dimerization. Two of the candidate molecules also have a biological effect, inhibiting formation of transformed cell foci by the Myc, Src, and Jun oncoproteins. These results provide proof of principle for the inhibition of protein-protein interactions by small, targeted molecules.

Binghe Wang
North Carolina State University
Fluorescent Tags Targeted on Cell-Surface Carbohydrates

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Malignant transformation is often associated with alteration of cell surface carbohydrates. The expression or over-expression of certain carbohydrates, such as sialyl Lewis X (sLex), sialyl Lewis a (sLea), Lewis X (Lex) and Lewis Y (Ley), has been correlated with the development of certain cancers. These cell surface carbohydrates can be used for cell-specific identification and targeting of carcinoma cells. The long-term goal of this project is the development of small molecule artificial receptors that can recognize target carbohydrate structures with high selectivity and affinity. Such receptors could be used for the development of fluorescent tags for cell-specific identification, tissue-specific imaging (such as MRI), and targeted delivery of therapeutic

agents. In this study, we target on sLex as the model carbohydrate and use colon cancer as the model biological system because the expression of sLex is often associated with the progression and metastasis of colon cancer. The short-term objective of this application is to develop tissue-specific fluorescent tags (sensors) that can recognize sLex with high affinity and selectivity. To this end, we have designed and synthesized about 20 potential sensor compounds. Their binding to sLex and other carbohydrates has been evaluated. Some promising leads have been identified. The structural features of these lead compounds will be further optimized in search of sensor compounds with high affinity and selectivity for sLex.

Yue Wang
The Catholic University of America
Intelligent Bioinformatics Approaches to

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Spotted cDNA microarrays are emerging as a powerful and cost effective tool for the large scale analysis of gene expression. To reveal the patterns of genes expressed within a specific cell essentially responsible for its phenotype, the profile of microarray expression is characterized through cluster discovery, gene selection, and class prediction. This presentation will report our progress in gene microarray data analysis using newly developed intelligent bioinformatics approaches. The discussion will entail five major issues: (1) Statistical modeling of gene expression profiles with a hierarchical standard finite normal mixture distribution; (2) Development of an unsupervised discriminative visual mining scheme to discover tumor clusters living in the molecular signature space; (3) Supervised evaluation of the intrinsic clusters produced by such scheme with phenotype-known microarray experiments; (4) Separability-based selection of the informative gene subset leading the biological process that generates the patterns; and (5) Preliminary consideration of an adaptive boosting of hierarchical subspace experts to microarray-aided molecular classification of cancer.

Heinz-Ulrich Weier
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Spectral Imaging for Phenotype Analysis of Cancer Cells

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This project seeks to develop a novel screening system to support cancer diagnosis, tumor staging, and prognostication based on the measurement of gene expression at

the RNA level. The underlying hypothesis is that tumors progress because of accumulation of genetic changes, and that the genetic changes manifest themselves in altered and measurable expression of one or several genes. Current techniques limit the measurement of RNA expression levels to a few genes per experiment. Sensitive tumor cell detection and prognostication will require methods that simultaneously profile the RNA expression levels of five or more suspected oncogenes/tumor supressor genes. Furthermore, if an oncogene is overexpressed, then its gene dosage should be determined as well. The quantitative analysis of several marker genes is crucial for sensitive tumor cell detection and accurate cell classification as well as the development of novel anti-tumor strategies. This information should help discriminate benign vs. malignant neoplasms and define prognostic markers.

Our appraoch is based on multicolor fluorescence *in situ* hybridization (FISH) in conjunction with Spectral Imaging (SIm). Existing SIm instrumentation can record fluorescence spectra from 400 nm to 1100 nm with about 10 nm resolution. This allows the unique labeling and detection of cDNA probes with commercially available fluorochromes. Spatial co-localization and spectral overlap is addressed by software processing of digitally recorded images, termed spectral un-coupling (SUN).
We used fluorescent beads in initial studies, and then selected cell lines based on their tyrosine kinase (tk) gene expression profiles. Our semi-quantitative studies using tk gene-specific cDNA microarray-based have already allowed us to define a set of cell lines and target genes for further studies.

We began to prepare fluorochrome-labeled cDNA probes to optimize the hybridization protocol and software algorithms. Artificial mixtures of cells from existing lines help us to determine the assay sensitivity, accuracy, and reproducibility. The work will be extended to thyroid and breast tumor tissues increasing the number of hybridization targets and correlating gene rearrangements/amplifications with gene expression. This technology, when applied to tissue from fine needle aspirates, could help diagnose and predict the course of suspicious cells in a rapid, inexpensive, and minimally invasive manner.

Sherman M. Weissman
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An Approach for Genome Wide Detection of Somatic Mutations in Cancer

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DNA glycosidases remove abnormal or mismatched bases from the sugar phosphate backbone of double stranded DNA. A number of glycosidases are known with different specificities for the base they remove. We have cloned and expressed fusion proteins of a human TG glycosidase, and a TG glycosidase from thermophilic bacteria, and also investigated the utility of E. coli MutY glycosidase. By use of these glycosidases we can obtain efficient cleavage of all single base mismatches in heteroduplex DNA. Mismatch containing duplexes can be trapped on beads containing immobilized glycosidases. By

this approach we have been able to obtain several hundred fold enrichment of a mismatched duplex from a perfect match duplex, contained in a complex DNA mixture. We have obtained similar enrichments for the perfect match duplexes.

To apply this approach to detection of somatic mutations in coding regions of mRNA in cancer as well as for other applications, we have designed protocols that meet certain critical criteria. The protocols begin with cDNA from two sources, such as normal cells and malignant cells from the same individual. We have designed adapters of similar or identical base sequences that make it possible to separately amplify either DNA duplexes in which both strands are derived from either the same source or duplexes in which the one strand is derived from each of the two sources. After ligation of appropriate adapters, this protocol makes it possible to mix restriction fragments from the two sources, denature, reanneal, and use glycosidase cocktails to separate the perfectly matched duplexes in the mixture from those containing internal mismatched bases. Each of the three types of duplexes (containing both strands from source A, one strand from each source, or both strands from source B) can be separately amplified and analyzed. An advantage of this approach is that variation in the efficiency of reannealing or mismatch recovery will not affect the relative recovery of duplexes of the different types. The recovered mismatch and perfect match duplexes can then be analyzed by gel display. In the future it may prove more convenient for global scanning of mismatches to use hybridization to cDNA, oligonucleotide, or genomic DNA arrays. In addition to the application to detection of somatic mutations, this approach has potential applications to several situations including detection of mutations in rare or sporadic Mendelian disorders as well as to the detection of induced mutations in inbred animals.

Kenneth T. Wheeler
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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. 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, 2. Using well-established mouse mammary adenocarcinoma cell lines 1 and termed 2 receptors that: (Wallen et al., 1984a, 1984b), we have demonstrated for:

- 2 receptors is higher in proliferative mouse mammary the density of denocarcinoma cells versus that of nonproliferative or quiescent mouse mammary adenocarcinoma cells (Mach et al., 1997). There was no difference in the density 2 receptors on a receptor/cell basis between diploid and aneuploid mouse of mammary carcinoma cells.
- the upregulation and downregulation of this receptor follows the kinetics of mouse mammary adenocarcinoma cells entering and exiting the cell cycle. The 2 receptor was found to kinetics of the up- and downregulation of the correspond to that of PCNA, a known marker of tumor cell proliferation (Al-Nabulsi et al., 1999).
- 2 receptor density between proliferative and the 10-fold difference in the quiescent mouse mammary adenocarcinoma cells grown in tissue culture also occurs in solid tumors growing in nude mice (Wheeler et al., 2000).
- 2 receptor agent (planar imaging studies with either a 99mTc-labeled 2 receptor agent (microPET imaging study) imaging study) or a 18F-labeled demonstrated a high tumor uptake and low background activity in regions such as 2 receptors in liver lung, heart, brain, and muscle. The high density of prevents the use of this approach for imaging tumors in the abdominal cavity.

We have observed the following for:

- 1 receptors was higher in proliferative mouse mammary?the density of adenocarcinoma cells versus that of nonproliferative or quiescent mouse mammary 1 receptors?adenocarcinoma cells (Mach et al., 1997). However, the density of on a receptor/cell basis and the P:Q ratio was higher in diploid versus aneuploid mouse mammary carcinoma cells.
- 1 receptors and ?there was a high correlation between the density of receptor mRNA in both the diploid and aneuploid mouse mammary adenocrcinoma cells;
- microPET imaging studies revealed a moderate tumor uptake of an 18F-labeled 1 receptor radiotracer and high background activity in lung, muscle, and brain. 1 receptor radiotracer in liver was much lower than?However, the uptake of the 2 receptor radiotracer that observed for the 18F-labeled receptors are a potential biomarker of cell.

These data suggest that proliferation in a variety of solid tumors. The high tumor uptake and suitable 2 receptor approach is predicted to target background ratios indicate that the be useful in measuring the proliferative status of tumors such as breast, brain, sarcomas, melanoma, lung, and head and neck tumors. The high density of receptors in liver prevents the imaging of tumors in the abdominal cavity. The 1 receptor low uptake of the 18F-labeled radiotracer in liver suggests that the imaging approach may be useful in imaging the proliferative status of tumors ?such as prostate and ovarian cancer. These data support the concept of using receptor-based radiotracers for assessing the proliferative status of solid tumors in vivo with radionuclide imaging techniques such as

PET and SPECT.

M. Wigler
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Detecting Gene Copy Number Fluctuations with Microarrays of Genomic

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Cancer can be thought of as a consequence of alterations of the genome, including translocations, amplifications, deletions and point mutations. These changes can be used as markers for the location of oncogenes or tumor suppressor genes. The understanding of the function of these oncogenes and tumor suppressors, once identified, can lead to improvements in therapy and diagnosis. We have developed a microarray-based method with utility in the detection of genome copy number fluctuations in cancer. In turn, these copy number fluctuations can be used to identify oncogenes and tumor suppressors. To overcome the low hybridization efficiency of the human genome, a complexity reducing "representation" of the genome is first prepared. A representation is a reproducible sampling of the genome, produced by first cleaving the genome with a restriction enzyme, such as BglII, ligation of adaptors to the fragment ends, and subsequent PCR. Amplification in the presence of many fragments results in preferential amplification of fragments in the size range of 200 to 1200 base pairs, typically a reduction in complexity to approximately 3% of the genome. Fragments isolated from a representation are arrayed, and the samples used for comparative hybridization are also representations, prepared from tumor and normal DNA sources. One major advantage of this format, is that representations can be prepared from as few as 1,000 cells enabling the use of minute specimens of clinical material.
We aim to construct a microarray of 30,000 probes to give a resolution of 1 probe every 100 kb. Since fragments are cloned randomly, they have no associated mapping information. In order to determine copy number fluctuations in the genome of cancer we must have mapping information for these probes. We have entered in collaboration with Dr. Richard McCombie, whose laboratory is sequencing the probes. The probes are being mapped and characterized by sequence using informatics. The probe sequence is broken into contiguous 16mers. The sequence of these 16mers are then compared to a database of in-silico BglII fragments from the golden path which have been similarly broken into contiguous 16mers. This identifies where the probe is on the golden path. In addition, from mer-frequency databases, we gain a measure of the repeat content of each probe.

Peter Wiktor
Engineering Arts
Automated Piezoelectric Pipetting Technology for DNA Analysis

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An automated pipetting instrument based on novel piezoelectric fluid transfer technology is being developed. A three axis servo system positions a head with eight individual pipetters to first intake fluid samples from a microtiter plate and then output the samples to another microtiter plate or onto a substrate like a glass microscope slide. The dispense volume is approximately 0.1 nanoliters making the instrument suitable for microarraying applications. An integral sensor checks if each intake and output task is accomplished successfully. A U.S. patent has been allowed and foreign patents have been applied for the fluid pipetting and sensing technologies. A U.S. patent application has also been filed for a compact, rugged housing for the device that completely protects the relatively fragile, enclosed piezoelectric and glass capillary tubes. Pipetting tasks are defined via drag-and-drop operations in a Windows based graphical user interface. The instrument control software detects if a pipetter fails and maintains a record of which intake and output tasks are completed successfully. If a given task fails then the software automatically commands a functioning pipetter to repeat it. The instrument can continue to function even if there are seven pipetter failures in a given run. Gene expression and SNP detection experiments will be run on this instrument at the Fred Hutchinson Cancer Research Center (Seattle, WA).

James C. Willey
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New Technology to Detect Genome-wide DNA Methylation Changes

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With recent completion of the human genome sequencing project greater attention is focusing on functional genomics. A key task now is to understand normal and pathological development and function by correlating gene expression pattern with known and newly discovered phenotypes. Progress in this area will accelerate greatly when there is an accepted common language for gene expression. Standardized RT-PCR (StaRT-PCR) allows rapid, reproducible, standardized, quantitative measurement of data for many genes simultaneously. An internal standard competitive template (CT) is prepared for each gene, cloned to generate enough for >109 assays and CTs for up to 1000 genes are mixed together. Each gene and reference gene is measured relative to respective CTs. Then each target gene is normalized to a reference gene to control for cDNA loaded into the reaction. Each gene expression measurement is reported as a numerical value that allows for direct inter-experiment comparison, for entry into a

common databank, and for the combination of values into interactive gene expression indices. As long as the same mixture of internal standard CTs is used, direct comparisons may be made among samples within the same experiment, different experiments in the same laboratory, and potentially different experiments in different laboratories.

In order to determine inter-laboratory reproducibility of gene expression measurement by StaRT-PCR, a blinded inter-laboratory study was conducted in which expression of ten genes was measured in a cDNA sample from human bronchogenic carcinoma cell line A549. The average coefficient of variation for the 9 quantifiable genes was 0.48 and all labs determined that expression of the tenth gene was too low to be measured. Analysis by capillary electrophoresis further increased precision, throughput, and ease of use of StaRT-PCR, and when combined with a novel, multiplex two-step StaRT-PCR method, decreased cDNA sample consumption by a factor of nearly 100 without loss of sensitivity to detect rare transcripts. Based on these results, StaRT-PCR provides standardized gene expression data suitable for research studies, clinical trials, and development of a gene expression databank and may be readily automated.

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Integrated Optical/Magnetic Resonance Microscopy for Cellular Research

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A combined optical (confocal) and magnetic resonance microscope (OM/MRM) is being developed for simultaneous studies of 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. At present this project is in its second year of the R21 phase, and the construction of the combined microscope is approaching its completion. In the R33 phase of this research, the utility and limitations of the integrated microscope will be evaluated by examining cells undergoing apoptosis, a process of critical importance to cancer therapy.

In this presentation the design of the instrument will be discussed and the first results, obtained with the combined microscope, will be shown. Specifically, the following issues will be addressed:

1 The layout of the instrument: The instrument utilizes an 89-mm diameter, vertical-bore Oxford magnet, operating at 11.7 Tesla, into which a bottom-loading OM probe and a top-loading MRM probe are inserted. Proton magnetic resonance images and spectra are acquired using a Varian, Unity-Plus imaging spectrometer.
2 The sample compartment. The sample chamber is part of a perfusion system, into which a horizontal cover slip, onto which cells are plated, can be inserted. The

optical field-of-view has a diameter of 2 mm, which means that maximal about 6,000-8,000 cells can be investigated. The temperature of the cells can be varied in the range 10-45 oC.

3 The confocal microscope. The objective is custom-made of non-magnetic materials, and has an in-plane resolution of maximal 1 micron, and a vertical resolution of about 33 microns.

4 The MR microscope. The sample compartment is surrounded by a gradient system, capable of producing gradients of 150-250 G/cm. The NMR coil is a small butterfly coil, mounted on top of the sample chamber. With this setup MR images of intra-cellular water in a cell monolayer are obtained with a resolution of 20 microns after about 4000 acquisitions, and proton NMR metabolite spectra of 6,000 cells with an adequate SNR are observed after 500 scans.

5 The image registration. Registration and calibration of the confocal and MR image spaces is obtained by permanently mounting into the sample chamber a m thick Kapton layer, placed near?fiduciary marker. This marker consists of 100- the top of the chamber, into which a micro-pattern is laser-etched.

6 First results. First combined images will be shown of the fiduciary marker and a monolayer of live Cho cells. Both a diffusion-weighted MR image and an image displaying the distribution of the diffusion coefficient of the intra-cellular water have been measured.

7 First applications. A method will be discussed to use the a priori knowledge provided by the high-resolution confocal images to improve the boundary resolution and the contrast in the MR images. Also, first results will be shown of the use of the combined microscope to monitor death in Cho cells during energy deprivation occurring when the perfusion medium is replaced by phosphate buffered saline.

8 Perspectives. Finally, potential applications of combined OM/MRM for cellular studies other than apoptosis will be discussed.

Michael E. Wright
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Quantitative protein profiling of androgen-responsive proteins in human prostate cancer: application of ICAT technology to a human disease model

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Comprehensive analysis of complex protein mixtures from biological samples has recently been accomplished by coupling microcapillary liquid chromatography (mLC) to tandem mass spectrometry (MS/MS) and database searching (Opiteck, Lewis et al. 1997; Link, Eng et al. 1999; Tong, Link et al. 1999). More importantly our laboratory has recently developed a new class of reagents termed isotope-coded affinity tags (ICAT) that selectively reacts and isotopically labels cysteine residues in proteins (Gygi, Rist et al. 1999). Combining the ICAT technology with mLC-MS/MS and database searching

has allowed us to simultaneously sequence identify and accurately quantify individual proteins within complex mixtures (Gygi, Rist et al. 1999). In this report we show how the ICAT technology was used to monitor quantitative changes in proteins isolated from the microsomal and nuclear fraction of androgen stimulated/starved LNCaP prostate cancer epithelia cells. We identified and quantified greater than ~1200 microsomal and ~ 800 nuclear proteins from LNCaP cells. Androgen stimulated protein expression of a number of enzymes involved in fatty acid biosynthesis, including fatty acid synthase (FAS), a well-documented target of androgen action in prostate epithelia (Swinnen, Esquenet et al. 1997). Androgen also stimulated protein expression of secreted and membrane-bound proteases, which included prostate-specific antigen (PSA), TMPRSS2, and kallikrein 2 (hK2).

Several families of previously characterized membrane receptors and proteins involved in adhesion, secretion and vesicular trafficking were also stimulated and/or repressed by the androgen hormone. Lastly, we successfully identified and quantified a number of nuclear proteins, which included the androgen receptor coactivator protein Transcription Intermediary Factor 2 (TIF2) (Voegel, Heine et al. 1996). A distinct group of previously uncharacterized coactivators, repressors, and tissue-specific transcription factors were also found to be targets of androgen regulation. We will integrate the results of this proteomic analysis with our cDNA array data and present a working model of how androgen controls multiple signal transduction pathways as part of the normal physiology of the prostate cell.

Fumiichiro Yamamoto
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New Technology to Detect Genome-wide DNA Methylation Changes

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Somatic epigenetic alterations in DNA methylation are tightly linked to development, cell differentiation and neoplastic transformation. For instance, hypermethylation of CpG islands in promoter regions has been found increasingly associated with transcriptional inactivation of tumor suppressor genes in carcinogenesis. Although techniques to determine the degree of methylation in specific DNA segments or in total DNA have been available, there are few techniques to efficiently scan and identify changes in methylation in the entire genome.

We have modified the Amplified Fragment Length Polymorphism technique and developed a method called Methylation Sensitive (MS)-AFLP. This PCR-based unbiased DNA fingerprinting technique permits the identification of the cleavage sites that exhibit DNA methylation alterations and subsequently allows the isolation of DNA fragments with these sites at their ends. Hyper/hypomethylation can be easily differentiated by the decrease/increase of band intensity, respectively. MS-AFLP requires low amounts of template DNA and electrophoresis of multiple samples in parallel enables easy identification of consistent common differences. NotI-MseI MS-

AFLP experiments using matched normal/tumor DNA have shown highly reproducible differences in banding patterns some of which were specifically linked to the tumor phenotype. Sequencing some of these bands has identified multiple numbers of homeotic genes and the genes involved in the regulation of homeotic gene expression. These results demonstrate the potential of MS-AFLP in identifying epigenetic alterations associated with cell differentiation and cancer.

We will transform this gel electrophoresis-based fingerprinting technique into a DNA microarray-based hybridization technique for general use of methylation alteration analysis of several biological problems. In the R21 phase, we will construct a pilot DNA microarray panel, examine the feasibility and sensitivity of several hybridization-based MS-AFLP and non-PCR methods using the pilot DNA microarray, and determine the best method(s) for further development. In the R33 phase, we plan to search for the prostate and breast cancer-specific DNA methylation alterations, using the selected method. We also plan to construct a cancer-specific DNA microarray for the clinical detection of DNA methylation alterations.

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Profiling Proteomes Using Multi-Dimensional Separations for Protein and Peptide Analysis

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A multidimensional HPLC system has been used to profile the yeast proteome. The method employs the shotgun analysis concept which entails a digestion of the protein components followed by separation and tandem mass spectrometry analysis. Proteins are then identified by searching tandem mass spectrometry data through protein sequence databases. By using a combination of ion exchange and reversed phase chromatography with tandem mass spectrometry, we show the system has less bias for classes of proteins typically not observed by 2-D gel electrophoresis. We observe acidic, basic, large and small proteins as well as membrane proteins. Elements of the experiment and results will be discussed.

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Molecular Beacons

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Molecular beacons are a class of oligonucleotides with a fluorophore at one terminus and a quencher at the other. The conformation of the molecular beacon in solution

effectively quenches fluorescence by resonant energy. Upon introduction of a target oligonucleotide, hybridization occurs resulting in the formation of a double helix. Molecular beacons with visible wavelength fluorescence are not suitable for in vivo applications because of excessive absorption and scattering by biopolymers. In order to detect specific mRNA in vivo with molecular beacons, we have prepared molecular beacons with fluorophores that absorb at near-IR wavelengths with different quenchers. The IRD-700 / rhodamine molecular beacons had significantly higher signal to noise ratios (25) relative to the IRD-700 / DABCYL molecular beacon (10) upon hybridization with target oligo. A signal to noise ratio of about 45 was observed for the IRD700/QSY-7 molecular beacon. QXY-7 was used for higher wavelength fluorophore, IRD-800 dye. IRD800/QSY-7 molecular beacon has 10-times fluorescence enhancement. We are developing novel quenchers to increase the signal to noise ratio for IRD-800 dye and seeing suitable transcripts for in vivo mRNA detection.

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Molecular Imaging Program: Targeting LDL Receptors in Tumors

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A number of tumors overexpress low density lipoprotein (LDL) receptors (LDLR). These include acute myelogenous leukemia (3-100-fold), colon cancer (6-fold), adrenal adenoma (8-fold), lung carcinoma, brain tumors and metastatic prostate cancer. These receptors are being exploited for the delivery of antineoplastic agents to these tumors as complexes with LDL or within lipid vesicles targeted to LDLRs by attachment of apoE. We are utilizing the same mechanism(s) to deliver to these tumors contrast agents that facilitate noninvasive imaging by near infrared (NIR) imaging.

LDL has been labeled with DiI (Molecular Probes, Inc.), a carbocyanine dye that absorbs and emits near 550 nm and with DiR, a tricarbocyanine dye that absorbs at ~740 nm and emits at ~780 nm. Confocal microscopy demonstrated that DiI-LDL bound to receptors on the surface of isolated B16 melanoma cells (which overexpress LDL receptors) at 40C and was internalized in these cells at 370C. Delivery of DiI-LDL following i.v. injection to in vivo B16 melanoma tumors implanted subcutaneously in nude mice was demonstrated by ex vivo redox scanning after rapid freezing (Quisdorff et al., Anal. Biochem. 148: 389-400, 1985). The dye was distributed throughout the tumor. The extent of in vivo intracellular localization of the dye is being determined by confocal microscopy of cells isolated from the tumor.

In vivo labeling of B16 melanomas and HepG2 hepatomas is also being monitored by

NIR imaging of DiR-LDL. In addition, three tricarbocyanine cholesterol lauroyl esters have been synthesized for reconstitution into LDL and delivery to -yl with ?LDLRs. These dyes were synthesized by reaction of 22-amino-5-pregnen-3 IRD41, IRD80 (LiCor, Lincoln, NB) and with a tricarbocyanine succinimide ester synthesized by Dr. Kai Licha.

We have also targeted liposomes containing indocyanine green (ICG) to LDLRs in the rat liver by adsorption of apoE to the surface of the liposomes. However, liposomes lacking apoE also exhibited a high level of binding to the liver, presumably through nonspecific association with the reticuloendothelial system (RES). Development of "stealth" liposomes that minimize nonspecific binding to the RES is planned.