

6th Principal Investigators Meeting Abstracts

6th Principal Investigators Meeting
Innovative Molecular Analysis Technologies Program
September 7-9, 2005
Ritz-Carlton
Washington, DC

Abstracts

Brian Balgley, Ph.D.
Calibrant Biosystems, Inc.

Integrated Top-Down/Bottom-Up Comprehensive Proteomics

*Balgley, Brian*¹

The R43 Phase of this project developed and demonstrated a capillary gel electrophoresis (CGE) based multidimensional protein separation platform, capable of providing significant analyte concentration and extremely high resolving power for handling complex protein mixtures prior to mass spectrometry detection. By coupling with a nanoscale trypsin membrane reactor, the ultrafast proteolytic digestion of proteins resolved and eluted from the CGE based separations enables the combined top-down/bottom-up characterization of post-translational modifications in complex proteomes.

The results of R43 Phase studies will allow validation of technology, and provide information necessary to guide the improvement, design, and completion of an automated, high throughput, robust, sensitive, and ultrahigh resolution proteome instrument using a multiplexing analysis format during the R44 Phase studies. The bioanalytical capabilities of CGE/CRPLC will be further expanded for processing membrane proteins, and will be able to rapidly identify and measure relative expression levels for thousands of proteins in a single analysis. Proteomic studies of steroid-activated programmed cell death during development of the fruit fly *Drosophila melanogaster* will serve as the model system for the evaluation of the technology.

Furthermore, the similarity between *Drosophila* and human cell death pathways indicates that many of the fly proteins identified in this study will be relevant to cell death in higher organisms. Studying the mechanisms of steroid-induced cell death will lead to a greater understanding of links between aberrations in steroid-regulated programmed cell death and human disorders including birth defects and cancer.

Annelise E. Barron, Ph.D.

Department of Chemical and Biological Engineering, Northwestern University

Fast Mutation Detection by Tandem SSCP/Heteroduplex Analysis on Microfluidic

Chips: 98% Sensitivity and Specificity

Annelise E. Barron, Ph.D¹, Christa N. Hestekin, B.S¹, David Y. Kim¹, Lionel Senderowicz¹

¹ Department of Chemical and Biological Engineering, Northwestern University
Evanston, IL

High-throughput genetic mutation detection technologies promise to revolutionize the diagnosis and treatment of cancer by enabling the correlation of prognosis with specific sequence alterations. We are developing one novel technological approach to screening for cancer-related mutations, based on microchip electrophoresis (ME). Mutations in the p53 gene, in particular, are known to be important in the pathogenesis of a variety of human cancers. Single-strand conformational polymorphism (SSCP) and heteroduplex analysis (HA) are two excellent and complementary electrophoretic methods for genetic mutation detection because of their simplicity, breadth of application, and low cost.

To create a clinically feasible mutation detection system, we have been working to optimize tandem-SSCP/HA for implementation on an ME platform by investigating the importance of variables such as polymer matrix properties, electric field strength, DNA sample stability and purity, etc. The R21-phase optimization of the method has been completed, and we recently performed a blinded study of over 100 genetic samples from exons 5-9 of the p53 gene.

Results show that our novel microchip electrophoresis based-SSCP/HA screening technology provided an overall sensitivity and specificity of **98%** for this set of samples from p53 exon 5-9. This high sensitivity and specificity as well as a rapid analysis time (< 10 min) for single-base mutations in the p53 gene exons 5-9 demonstrates the powerful clinical potential of ME-SSCP/HA.

C. Fred Battrell, Ph.D

Microcytometer Lab CArD for Detection of Rare Cancer Cells

No abstract available

Jesse S. Buch, Ph.D.

Calibrant Biosystems, Inc.

Gel Protein Extraction Platform for Protein Identification

No abstract available

Pierre Chaurand, Ph.D.

Vanderbilt University

Profiling and Imaging Mass Spectrometry on Thin Sections from Solvent Preserved Tissue Specimens

Pierre Chaurand, Ph.D¹, Kirk B. Lane¹, Richard M. Caprioli¹

¹ *Vanderbilt University, Nashville, TN*

Over the last years, methodologies have been developed for the direct analysis and imaging of tissue biopsies by MALDI MS [1]. Efforts have been focused on fresh frozen tissue sections with the hypothesis that minimal protein degradation occurs to the tissue biopsy when frozen. Although ideal to work with from a MS point of view, frozen tissues have several drawbacks—namely, transport and long-term storage. Second, pathologists prefer to make their diagnostic evaluations on sections from fixed tissues, which present more key features than fresh frozen sections. In this respect, sections from formaldehyde (formalin) fixed tissues are usually preferred. These, however, present an enormous challenge from a mass spectrometry perspective. Indeed, the crosslinking chemistries occurring between formaldehyde and the biomolecular content of the tissues are poorly understood and not easily reversible, a step absolutely critical for subsequent intact protein MS analyses. Solvent preserved tissues, however, present the triple qualities of 1) long-term preservation at room temperature, 2) generation of high-quality histological sections, and 3) no chemical alteration of the proteins. We present here the first report of analyses by MALDI MS imaging of thin sections from solvent preserved mammalian tissue specimens.

Mouse liver, lung, and lung tumors (induced with Lewis lung carcinoma cells) were either preserved by snap freezing in liquid nitrogen or by dehydration (fixation) with graded ethanols and xylene. After fixation, the tissue samples were embedded in paraffin at 65°C and stored at room temperature. Ten micron thick sections were cut from all samples using a cryostat (fresh frozen) or microtome (ethanol preserved), and mounted on MALDI compatible conductive glass slides. Paraffin removal from ethanol fixed sections was accomplished by washes in xylene and graded ethanols. All sections were analyzed by MALDI TOF MS using sinapinic acid as matrix .

Comparative MALDI MS experiments were performed between fresh frozen and ethanol preserved mouse liver, lung, and Lewis lung carcinoma cell-induced lung tumors. In all cases, protein signals were observed with about 50% peptide and protein signal homology between the fresh frozen and ethanol fixed samples. Some general trends were also observed. More low MW signals (below $\sim m/z$ 4800) were observed from the ethanol preserved section. More high MW signals (above $\sim m/z$ 20,000) were observed from the fresh frozen section. The protein profiles and images recovered upon analysis of ethanol preserved normal lung tissue sampled in a control mouse were compared to the ethanol fixed Lewis lung carcinoma cells and a lung tumor as well as the surrounding tissue adjacent to the tumor from a mouse injected with Lewis lung carcinoma cells. Significant differences in signal expression were observed between the control section and the tumor. Second, numerous protein signals observed when analyzing the cancer cells alone were detected when analyzing the tumor section. The profiles also indicate that the lung tissue surrounding the tumor nodules possess characteristics closer to the tumor than that of normal lung tissue. After homogeneous matrix coating, high-quality images were also obtained for multiple proteins from ethanol preserved cancerous lung sections. Both lung-specific and tumor-specific protein markers were detected. Several protein markers unique to the different tissue types

forming the upper respiratory airways were also imaged. Finally, we have also found that the MALDI MS analysis of the same tissue block of normal lung within a 6-month lapse time showed very similar protein signals.

Kevin P. Claffey, Ph.D.
University of Connecticut Health Center

Identification of Immune-Selected Breast Cancer Antigens

Kevin P. Claffey, Ph.D.¹, Jason J. Sparkowski¹, Martin P. Keough¹, Frank Vumbaca¹, Katherine Neurath¹, Melinda Sanders¹, Scott H. Kurtzman¹

¹ *1Center for Vascular Biology, Departments of Cell Biology, Anatomic Pathology and Surgery, University of Connecticut Health Center Farmington, CT*

Immune-dependent responses are selective in defining non-self or aberrant antigen presentation. Unfortunately, long-term antibody production to human cancer antigens is limited. In an innovative and novel approach, we have developed a method to utilize primary immune reactions in tumor draining lymph nodes to provide the means to identify biologically active tumor antigens originating from breast cancers. This novel approach combines a series of steps to coordinate the construction of low complexity antibody cDNA libraries and protein production that are used to identify tumor antigens using sensitive antibody microscale “antigen-trap” assays followed by LC-MS/MS antigen identification.

A representative and novel breast cancer antigen has been identified as the cell surface glycoprotein, stromal cell derived receptor-1 (SDR-1) and shown to be upregulated in human breast epithelial tumors at the mRNA level, produced as an alternative protein isoform (45kDa) in tumor lysates, and high level expression associated with highly invasive tumor types and over-expressed in 50% of tumors representing distal metastatic disease. Additional tumor antigens will be identified and verified using a medium throughput platform and secondary biochemical, molecular, and immunological verification assays.

This project applies innovative technologies that demonstrate: a) tumor-draining lymph nodes are immune-reactive to aberrant breast cancer antigens and produce antigen-dependent somatic hypermutation in proliferative B-cell germinal centers, b) that antigen-binding domains of somatic hypermutated antibodies synthesized as recombinant VH and/or VH-VI/Vk scFv proteins can specifically recognize and identify breast cancer antigens, and c) antigens identified can be verified as diagnostic for breast cancer sub-phenotypes.

Xiang-Dong Fu, Ph.D.
University of California, San Diego

Typing the Transcriptom of Prostate Cancer by Splicing Array

Xiang-Dong Fu, Ph.D.¹, Hairi Li¹, Jessica Wang-Rodrigues², Jian-Bing Fan³

¹Department of Cellular and Molecular Medicine, ²Department of Pathology, University of California, San Diego, La Jolla, CA, 3Illumina, Inc., San Diego, CA

Most genes in mouse and human are now known to undergo alternative splicing, which may significantly contribute to the complexity of the proteome in eukaryotic cells. Because the expression of specific mRNA isoforms reflects an integrated outcome of both transcriptional and post-transcriptional regulation, the profile of mRNA isoforms may serve as more robust molecular signatures for the biological state of a cell than total transcript abundance.

To explore this idea in understanding the regulation of gene expression and apply the approach to cancer classification, we constructed a sensitive microarray system to interrogate the expression of a comprehensive list of genes previously implicated in prostate cancer. A data analysis package was coordinately developed, permitting for the first time to calculate differential expression of both total transcript and specific mRNA isoforms in prostate cancer cell lines and tumor samples. Using this novel approach, we showed that individual cancer cell types could readily be characterized by unique patterns of mRNA isoform expression. The novel “two-dimensional” profiling strategy revealed extensive coupling between transcription and splicing in individual prostate cell lines, a subset of which was regulated by androgen. The data were extensively validated by independent microarray and RT-PCR methods.

Taking advantage of the unique power of our technology in profiling RNA from formalin-fixed, paraffin-embedded tissues, we then applied the technology to prostate cancer samples and identified a highly diagnostic panel of mRNA isoform signatures for both prostate cancer tissues and cell lines, which were cross-analyzed by using independent sets of data for feature selection and validation. Interestingly, the molecular signatures deduced from prostate tumors could be used to characterize prostate cancer cell lines by unsupervised clustering analysis, but the converse was not true, suggesting that prostate cell lines both retained molecular signatures from original tumors and gained additional molecular alterations during *in vitro* passages. Finally, representative mRNA isoform biomarkers were validated by laser capture microscopy. Together, our results demonstrate the robustness of the technology and soundness of the data analysis, poising the system for large-scale applications in cancer research.

Kyle A. Furge, Ph.D.

Van Andel Research Institute

Identification of Cytogenetic Abnormalities Using Gene Expression Data

Kyle A. Furge, Ph.D.¹, Karl J. Dykema¹, Coral Ho², Xin Chen^{2,3,4}

¹Laboratory of Computational Biology, Van Andel Research Institute, Grand Rapids, MI,

²Department of Pharmaceutical Sciences, ³Cancer Center, ⁴Liver Center, University of California, San Francisco, CA

Aneuploidy is a common feature of cancer and several lines of evidence suggest that

cytogenetic aberrations correspond with differences in tumor diagnosis, prognosis and treatment. While molecular genetic based methods, such as comparative genomic hybridization (CGH), have traditionally been used to determine cell karyotypes, recent transcriptional profiling studies have suggested that it is possible to predict cytogenetic changes from microarray gene expression data.

For example, if a region of the genome is amplified, often the majority of genes that map within the amplified region show increased expression when compared to genes located in cytogenetically normal regions. As such, these regional expression biases (REBs) can act as surrogates for cytogenetic data obtained using other molecular technologies. We have developed computation approaches to scan gene expression data to identify likely cytogenetic abnormalities at ~5-10 megabase resolution.

As a proof-of-principle, REBs were identified from a set of hepatocellular carcinoma (HCC) gene expression profiles using a multiple span moving binomial test and compared to genetic abnormalities identified using array-based comparative genomic hybridization (aCGH). In the majority of cases, REBs overlapped genetic abnormalities as determined by aCGH. As such, identification of REBs can provide reasonable approximations of cytogenetic abnormalities.

In addition, the prevalent overlap of transcriptional and cytogenetic abnormalities support tumorigenesis models that advocate recurrent cytogenetic aberrations, via their significant influences on gene expression, play important roles in pathogenesis. We are now correlating individual gene expression changes to regions of inferred cytogenetic gain/loss to identify likely candidate tumor modifying genes.

Mariano A. Garcia-Blanco, M.D.
Ph.D. Duke University Medical Center

Imaging Alternative Splicing in Tumors and Tissues in Living Animals

No abstract available

Sandra M. Gaston, Ph.D.
Harvard Medical School

Tissue Print Micropeel and Multi-Enzymatic Tissue

Sandra M. Gaston, Ph.D.¹, Melissa P. Upton, M.D.¹, Dang Vu¹, Ting Ting Fu¹, Tendai Chizana¹, Dana L. Goldner¹, Robert E. Lenkinski, Ph.D.¹

¹Beth Israel Deaconess Medical Center and Harvard Medical School Boston, MA
To realize the full potential of a wealth of new tumor biomarker information, it is essential to develop strategies for profiling human tissue and tumor specimens that are workable in a clinical setting. We have developed a set of novel "tissue print" techniques that allow us to profile the molecular markers over extended areas of human tissue and tumor samples without damaging the specimen (Gaston et al. Nature Medicine 11(1):95-101, 2005). "Tissue printing" transfers cells and extra-cellular matrix components from a tissue surface onto nitrocellulose membranes, generating a two-dimensional anatomical image on which molecular markers can be visualized by specific protein and RNA/DNA detection techniques. The resulting marker maps can then be superimposed directly onto histopathological and radiological images of the

specimen, permitting molecular identification and classification of individual malignant lesions.

In the clinical setting, tissue print techniques simplify the process of obtaining an adequate representation of human cancers in biopsies and surgical specimens, particularly when the tissue of interest must be conserved for diagnostic evaluation. Moreover, when the molecular profile of the specimen is itself of potential diagnostic importance, such as in the evaluation of surgical margins for microscopic tumor, the tissue-printing platform can be adapted as a clinical tool in order to provide “molecular sections” of the specimen. Tissue printing thus provides both a new strategy for the discovery and validation of tumor biomarkers and a practical approach to the application of molecular profiling to important clinical problems in surgical oncology.

Our long-term goal is to utilize our tissue print techniques as a platform technology to simplify the process of obtaining representative high-quality samples of human cancers in biopsies and surgical specimens. To achieve this goal, we are developing protocols for this tissue-sampling platform that support both proteomic analysis and PCR based DNA and mRNA profiling techniques. Our current IMAT R21 project is focused on optimizing protocols for preparing mRNA and DNA from tissue prints collected from human prostate and breast tissue/tumors specimens in order to define quantitative standards for yield and reproducibility. These protocols will provide the foundation for advancing our tissue print molecular profiling technologies onto an automated platform in the R33 phase of this project.

Sandra M. Gaston, Ph.D.
Harvard Medical School

Tissue Print Micropeel and Multi-Enzymatic Tissue Liquefaction (MELT) Technologies Combine to Create an Efficient, Scalable Platform for Profiling RNA and DNA Biomarkers in Human Tissue Biopsies and Surgical Specimens

Sandra M. Gaston, Ph.D.¹, Melissa P. Upton, M.D.¹, Dang Vu¹, Ting Ting Fu¹, Tendai Chizana¹, Dana L. Goldner¹, Robert E. Lenkinski, Ph.D.¹

¹Beth Israel Deaconess Medical Center and Harvard Medical School Boston, MA,

²Ambion, Inc., Austin, TX

One of the ultimate goals of current efforts to identify and validate new tumor markers is to deliver on the promise of “personalized” medicine for patients diagnosed with cancer. Sample preparation technologies serve as a foundation for the successful translation of state-of-the-art molecular profiling of human cancers from the basic research centers into multi-center clinical trials and ultimately into general clinical application. While careful management of sample quality is essential for all types of biomarker analysis, collecting and processing samples suitable for analysis using RNA-based tumor markers present a particular challenge in most clinical settings.

Recently, we linked two IMAT-supported technologies to produce a general platform for

collecting and processing “micropeels” of human tissue specimens for RNA and DNA biomarker profiling. The tissue print-MELT sampling system overcomes two major obstacles in the preparation of clinical tissue samples for RNA-based molecular profiling: the need to preserve the integrity of the specimen for clinical diagnosis and general lack of facilities to “snap freeze” tissue immediately upon resection. Micropeel samples of cells and extracellular matrix are transferred from human specimens onto nitrocellulose membranes using tissue print technologies developed by the Gaston laboratory at Beth Israel Deaconess Medical Center in Boston.

Tissue print collection does not compromise the specimen for clinical histopathological diagnosis or for other more tissue-intensive studies. RNA and DNA are extracted from the cells transferred to the nitrocellulose by immersing tissue-print segments into a MELT solution formulated by the Latham group at Ambion. Unlike conventional RNA isolation reagents, MELT enzymes rapidly solubilize cells from the micropeel and irreversibly degrade nucleases, stabilizing both the RNA and DNA for downstream isolation and analysis.

Our preliminary results indicate that the tissue print-MELT sampling system is generally applicable to many different types of human specimens. Optimal conditions for specific tissue and tumors and for specific downstream applications are currently in development.

David R. Goodlett, Ph.D.
University of Washington

Increased Protein and Proteome Coverage from Novel Software to Interpret Peptide Tandem Mass Spectra Acquired in a Data-Independent Fashion Via Shotgun CID

David R. Goodlett, Ph.D.¹, Catalin Doneanu¹, Alexander Yates¹, Soyoung Ryu¹, Gregory K. Taylor¹, Erik Nilsson², Brian Pratt², Dragan Radulovic³

¹Department of Medicinal Chemistry, University of Washington, Seattle, WA, ²Insilicos LLC, Seattle, WA, ³Florida Atlantic University, Boca Raton, FL

Collision induced dissociation (CID) of peptides was conducted in parallel in a data independent manner as peptides co-elute from a reversed-phase C18 based separation into an electrospray ionization (ESI) source coupled to a quadrupole-time-of-flight mass spectrometer. We refer to this as shotgun or parallel CID as opposed to traditional serial CID conducted in a data-dependent manner. The result of co-fragmentation of many peptides simultaneously is loss of parent-fragment ion genealogies that are maintained during standard serial CID of peptides. We developed software to sort which fragment ions belong to a given parent based on chromatographic retention times. This software allows traditional .dta files used for SEQUEST based sequence database searching of peptide tandem mass spectra to be constructed. Like a .dta file for serial data each shotgun based .dta file contains a known parent mass and a list of fragment ion masses. We tested our software on a series of proteomic samples of increasing

complexity. Not surprisingly, we found that samples of limited complexity showed higher individual protein sequence coverage when analyzed by shotgun CID versus serial CID. As sample complexity increased individual protein sequence coverage decreased, but proteome coverage increased.

The shotgun CID data processing algorithm utilizes the following workflow: 1) Obtain a series of alternating low and high energy CID scans; 2) For a moving window of low energy scans t seconds wide, where t is the nominal width of a chromatographic peak: Average the scans together, then perform de-siotoping and charge state de-convolution to obtain a list of singly protonated “precursor” masses; 3) For the corresponding time window of high energy scans, average the scans together to create a “shotgun tandem” scan; 4) For each “precursor” mass above a given intensity threshold, match the chromatographic profile of the precursor to the profile of each potential “child” ion in the high energy scan; and 5) For each child ion that matches the parent profile within a given tolerance, add that child to the “tandem” scan then output a .dta file with that precursor mass and the contents of the “tandem” scan.

Shotgun CID proved to be superior to serial CID in all cases examined. Shotgun results, like those from the five and ten protein mixtures, were dramatically better than those from serial analysis with ~ two-times more unique peptides identified in each case. While the shotgun CID software pipeline achieved much better results than serial data analyzed by SEQUEST for the relatively simple 5 and 10 protein mixtures, it failed to achieve significantly better results on the whole cell yeast lysate. We feel that this failure to achieve very much better results is in part due to the increase in rate of false positive identifications for shotgun CID. Thus, while we have made significant progress since our initial report [3] by developing and automating the shotgun CID software pipeline presented here, additional software implementations are required to increase the information extracted from shotgun CID. For example we are in the process of incorporating peptide retention time prediction into the identification process and peptide mass fingerprinting to thoroughly exhaust the available information.

Roland D. Green, Ph.D.
NimbleGen Systems, Inc.

Mapping Regulatory Pathways in Cancers

Roland D. Green, Ph.D.¹, Bing Ren², Eirikur Steingrímsson³

¹ NimbleGen Systems, Inc., Madison, WI , ² Ludwig Institute for Cancer Research, University of California, San Diego, School of Medicine, La Jolla, CA , ³ Iceland Genomics Corporation, Reykjavik, Iceland

Many different transcription factors (TFs) have been found to be involved in key pathways in cancer. A better understanding of the regulatory pathways and gene targets governed by these TFs in normal and neoplastic cells will lead to the discovery of additional cancer genes which will provide new therapeutic targets. Also, a comprehensive map of the targets and binding sites of TFs is a necessary step in the

delineation of the wiring diagram of the cell. It is also possible that the target binding patterns of TFs in cancer cells could provide a powerful new method of classifying and diagnosing tumors.

To map TF binding sites in the human genome, we as well as have begun using the emerging technology of chromatin immunoprecipitation (ChIP) on microarrays (ChIP chip). We developed a new microarray set that contains 15 million probes on 38 arrays that tile through the entire human genome with 50mer probes every 100 bases. This array set offers the potential for groundbreaking studies of TF binding sites. However, large-scale studies will be expensive given the current state of the technology. For the R21 phase of this grant, we propose to refine and implement two cost reduction protocols, array reuse and 4-color hybridization; so that these genome wide ChIP chip studies will become practical for large numbers of laboratories. As a model system, we will study the binding patterns of the TF TCF4 in colon cancers for the reuse, 4-color and genome wide tiling experiments.

Martin Guthold, Ph.D.
Wake Forest University

Novel, Single-Molecule Methodology to Identify New Aptamers

Martin Guthold, Ph.D.¹, Lu Peng¹, Roger Cubicciotti²

¹Department of Physics, Wake Forest University, Winston-Salem, NC , ²Nanomedica, Inc., Newark, NJ

We are developing a novel, single-molecule methodology to identify aptamer molecules in a pool of random oligonucleotides. Unlike SELEX, our approach does not require iterative cycles of selection and partitioning, but aptamers are selected one-by-one. At the core of our methodology is unique instrumentation that combines a nanoManipulator Atomic Force Microscope (nM-AFM) and an inverted optical microscope with TIRF illumination and FRET detection capabilities.

This instrument, in combination with PCR, is used to identify new aptamers as follows. Target molecules are labeled with a donor fluorophore and linked to a cover slip. Random-sequence oligo libraries are labeled with an acceptor fluorophore and a 10 nm bead (for AFM detection and pick-up). The oligos are then flowed over the target area. High-affinity, target-specific aptamers will bind tightly to the target for relatively prolonged periods resulting in a strong fluorescence signal. After a fluorescence signal has been observed with the optical microscope, the nM-AFM is used to obtain a high-resolution image of the fluorescence signal-generating region. Subsequently, the AFM tip is used as a “gripper” to retrieve the bead plus the attached aptamer. PCR is then used to amplify the extracted aptamer.

We have implemented the instrumentation and completed proof-of-principle experiments with a known thrombin aptamer. We successfully detected (AFM and fluorescence), extracted and amplified thrombin-aptamers from a pool of random

oligonucleotides.

Brian B. Haab, Ph.D.

Van Andel Research Institute

Longitudinal Cancer-Specific Serum Protein Signatures

Brian B. Haab, Ph.D.¹, Songming Chen¹, Richard Hay¹, Ziding Feng², Robert Vessella³, William Catalona⁴

¹*Van Andel Research Institute, Grand Rapids, MI*, ²*Fred Hutchinson Cancer Research Center, Seattle, WA*, ³*University of Washington Medical Center, Seattle, WA*,

⁴*Northwestern University, Chicago, IL*

Serum markers hold great promise for improving the care and treatment of cancer patients. Although many proteins have serum levels associated with various cancers, each has limited clinical usefulness when measured individually at single-time-points. The lack of sensitivity and specificity of current serum markers stems from heterogeneity in the baseline levels of the marker proteins and heterogeneity in the tumors and patients. A biomarker discovery strategy that accounts for the heterogeneity in people and tumors is to use individualized thresholds, based on longitudinal measurements, to precisely define abnormal levels for each individual.

Previous research has shown that biomarkers defined by longitudinal measurements could have greatly improved specificity and sensitivity over current markers. No systematic study of this topic has been performed, largely because of the lack of a convenient technology for that purpose. A well-developed and validated antibody microarray technology in the laboratory of Dr. Haab now makes this exploration possible. The multiplex detection capability of the antibody microarray will allow us to test the performance improvement upon using longitudinal measurements for many different proteins, and to establish the general principles that define the use of longitudinal markers.

To test this strategy, we will evaluate the sensitivity, specificity and time of the detection of prostate cancer recurrence using both longitudinal and single-time-point measurements of many different prostate cancer-related proteins in serum. This new approach addresses fundamental issues in biomarker research and should result in valuable information for a wide variety of research areas.

Tomasz Heyduk, Ph.D.

St. Louis University School of Medicine

Molecular Beacons for Protein Detection

Tomasz Heyduk, Ph.D¹, Ewa Heyduk¹, Eric Knoll¹

¹*Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine St. Louis, MO*

The goal of this project is to develop novel nucleic-acid based sensors designed to produce fluorescence signal upon recognition and binding to the target protein. In the first phase of the project model protein systems were used to provide “proof of principle” verification of molecular beacon design for proteins exhibiting natural sequence-specific DNA binding activity. Experiments with several DNA binding proteins under variety of solution conditions provided ample evidence for general feasibility of our assay. We proposed and experimentally verified a physical model describing the behavior of the assay. Various variants of the original design and various modes of generating the fluorescence signal detecting the presence of the target protein were tested. Optimized beacons were able to detect the target protein at subnanomolar concentrations, in the presence of large excess of unrelated proteins and in cellular extracts.

Additional experiments verified compatibility of the assay with high-throughput detection methodologies. In the next step we generalized our molecular beacon design to include detection of proteins lacking natural DNA binding activity. We used thrombin and thrombin-specific aptamers as a model system to test this design. We have tested several variants of thrombin beacon differing in the mode by which fluorescence signal reporting the presence of the target protein was generated. The optimized beacon was able to detect thrombin in picomolar concentrations, in the presence of large excess of unrelated proteins, in plasma and in cellular extracts.

All the milestones of the R21 phase of the project were accomplished, leading to a successful transition to R33 phase in which molecular beacons detecting several cancer-related target proteins will be developed.

David Hill, Ph.D.
Harvard Medical School
Interactome Networks

David Hill, Ph.D.¹, N. Ayivi-Guedehoussou¹, N. Bertin¹, M. Boxem¹, M. Cusick; M. Dreze¹, A. Dricot¹, D. Dupuy¹, T. Hao¹, T. Hirozane-Kishikawa¹, N. Klitgord¹, P. Lamesch¹, N. Li¹, Q. Li¹, S. Milstein¹, J. Rosenberg¹, J-F. Rual¹, C. Simon¹, A. Smolyar¹, M. Tewari¹, K. Venkatesan¹, and M. Vidal¹

¹Center for Cancer Systems Biology and Department of Cancer Biology, Dana-Faber Cancer Institute, and Department of Genetics, Harvard Medical School Boston, MA

Despite the considerable success of molecular biology to understand diseases such as cancer, many fundamental questions remain unanswered. Most importantly, since the majority of gene products in the cell mediate their function together with other gene products, biological processes should be considered as complex networks of interconnected components. In other words, for any normal biological process, or any disease mechanism, such as cancer, one might consider a “systems approach” in which the behavior and function of such networks are studied as a whole, in addition to studying some of its components individually. The draft of the human genome sequence is likely to help such a transition from molecular biology to systems biology.

Our laboratory uses a model organism, the nematode *C. elegans*, to study the role of protein networks in development and, doing so, develop the concepts and technologies needed for a transition to systems biology.

Our goals are to:

- i Generate protein-protein interaction or “interactome” maps for *C. elegans* networks involved in development.
- ii Develop new concepts to integrate such interactome maps with other functional maps such as expression profiles (transcriptome), global phenotypic analysis (phenome), localization of expression projects (localizome), etc.
- iii Use such integrated information to discover novel network properties.

Eugene S. Kandel, Ph.D.
Cleveland Clinic Foundation

Improved Methods of Insertional Mutagenesis for Forward Genetics in Mammalian Cells

Eugene S. Kandel, Ph.D.¹, Tao Lu¹, Youzhong Wan¹, Maupali Dasgupta¹, Mukesh K. Agarwal¹, Mark W. Jackson¹, Patrick Varley¹, and George R. Stark¹

¹*Cleveland Clinic Foundation Cleveland, OH*

Genetic dissection of signaling pathways in mammalian cells involves screening or selecting phenotypic mutants obtained by a variety of techniques. The concerns about current methods include inadequate genome coverage and difficulty in validating the link between mutation and phenotype. We show that the ability to induce mutations increases greatly if a randomly inserted promoter directs transcription into the host DNA. We predict that either gain-of-function or loss-of-function mutants could be obtained depending on the position and orientation of an insert. The mutant phenotype is due to the expression of a hybrid transcript derived from the vector and the insertion site. Since other alleles of the affected gene remain intact, the phenotype is dominant, but reversible by inactivating the promoter, for example by site-specific recombination. Importantly, in mutant clones with multiple inserts, limited excision yields progeny with different patterns of inserts remaining. Characterizing these progeny allows the mutant phenotype to be associated with a specific target gene.

We have used this technique to search for proteins that regulate NF- κ B-dependent signaling in human cells. Reversible mutants were readily obtained using either retroviral or transposon-based vectors and currently are under investigation. Examples of targeted genes include *relA*, which codes for the NF- κ B p65 subunit, and the NF- κ B regulator *act1*. Overexpression of the corresponding proteins, caused by insertion of a promoter into the first intron of each gene, leads to NF- κ B-dependent secretion of factors that activate NF- κ B through cell-surface receptors, establishing an autocrine loop.

Relative simplicity and robust target validation make the method suitable for a broad

range of applications. We are currently adopting our mutagenesis strategy for use in animal models

Shana O. Kelley, Ph.D.

Boston College

Nanoscale Electrocatalytic Protein Detection

No abstract available

Raymond A. Kim

Ph.D., GenePrism, Inc.

A Multiplexed Protein Measurement Technology Using Single-Antibody, Signal Amplified Detection Method

No abstract available

Daniel R. Knapp, Ph.D.

Medical University of South Carolina

Monolithic Column Plastic Microfluidic Device for Peptide Analysis Using Electrospray from a Channel Opening on the Edge of the Device

No abstract available

David B. Krizman, Ph.D.

Expression Pathology, Inc.

Proteomic Profiling of Fixed Tissue Archives by Mass Spectrometry

David B. Krizman¹

¹*Expression Pathology, Inc., Gaithersburg, MD*

The ability to conduct proteomic experiments with formalin fixed archival tissue would allow for interrogation of large collections of human and animal tissues with well-documented pathological, toxicological, and clinical outcomes. However, chemically-induced protein cross-linking currently limits large scale proteomic analysis of fixed tissue. Expression Pathology Inc. has developed a simple and efficient sample preparation methodology (termed Liquid Tissue™) for extraction of soluble and dilutable protein/peptides directly from fixed tissue that has been further optimized for proteomic analysis by mass spectrometry.

The Liquid Tissue™ - MS method offers the capability to analyze and identify unique peptides and proteins from fixed archival tissue on a variety of mass spectrometry platforms including SELDI-TOF, MALDI-TOF/TOF, and LC-ESI-MS/MS. Identification of unique proteins in the range of 400 to 1,200 per sample is routinely achieved, and a wide variety of fixed cancer tissue types that range from 1 month to 15 years post-fixation have been used equivalently for expression analysis of known cancer biomarkers . In-depth analysis of prostate tissue indicates expression of known prostate cancer markers such as PSA, PAP, and PEBP in prostate cancer (PCa), as well as differential expression of specific proteins between PCa and benign prostatic

hyperplasia (BPH).

These results illustrate novel proteomic technology to identify and associate specific protein biomarkers with the pathological manifestation of disease through large-scale and complex proteomic analysis of formalin fixed tissue archives.

Stephen Kron, M.D., Ph.D.
The University of Chicago

Solid Phase Tyrosine Kinase Activity Assays for Clinical Use in Leukemia Diagnosis, Therapeutic Planning, and Monitoring

*David B. Krizman*¹

*Stephen Kron, M.D., Ph.D.*⁴, *Wendy Stock*², *Stephen Kent*³, *Sean Palecek*⁴, *Ezra Abrams*⁵, *Ding Wu*¹, *Dorie Sher*², *Laurie Parker*³, *Shawn Brueggemeier*¹, *Jennifer Campbell*¹, *Evan Nair-Gill*⁴, *Matthew Myers*², *Mariah Siddiqui*², and *Emily Testa*³

¹*Center for Molecular Oncology and Departments of*²*Medicine and*³*Biochemistry & Molecular Biology, The University of Chicago, Chicago, IL,*⁴*Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI,*⁵*Matrix Technologies Corporation, Hudson, NH*

The primary goal of CA103235, “Bcr-Abl kinase assays for STI571 sensitivity or response,” is to develop quantitative assays to measure Bcr-Abl tyrosine kinase activity and inhibitor sensitivity in cell extracts. This effort is stimulated by the recent development of Imatinib (STI571) and newer, more potent Abl kinase inhibitors. These ATP analogs target Bcr-Abl, an oncogenic kinase that results from the t(9;22) reciprocal translocation characteristic of CML and some ALL. Typically, Imatinib induces remission in CML, but patients may develop resistance, leading to recurrence.

We are developing a clinical assay for Bcr-Abl activity and inhibition to guide therapy. Our approach has been to incubate immobilized protein and peptide Bcr-Abl substrates with cell extracts and ATP, with or without inhibitors, and quantitate specific tyrosine phosphorylation. We have investigated several substrate immobilization options and compared detection methods including incorporation of radionuclides, phosphotyrosine immunoreactivity and MALDI-TOF mass spectrometry. Our progress has contributed to three papers published or in press and to patents applied for and in preparation.

Year one of the development phase has led to validation of high sensitivity and specificity Bcr-Abl substrates and their use in bead-based Bcr-Abl assays appropriate for analysis of clinical material. Initial patient studies are underway. We have recently adapted our approach to commercially available ez-rays™ hydrogel-coated multiwell plates to facilitate high-throughput screening for new Bcr-Abl inhibitors using cells or cell extracts. Finally, we have pioneered a novel MALDI-TOF detection strategy amenable to highly multiplexed kinase assays that will serve as a foundation for further development.

Joshua LaBaer, M.D., Ph.D.,
Harvard Medical School Institute of Proteomics
“Harnessing the Human Proteome”

David B. Krizman¹

Joshua LaBaer, M.D., Ph.D.

¹*Harvard Medical School Institute of Proteomics, Cambridge, MA*

One of the most compelling steps in the post-genomic era will be learning the functional roles for all proteins. The Harvard Institute of Proteomics (HIP) has initiated a project to create a sequence-verified collection of full-length cDNAs representing all human coding regions in a recombinational vector system that allows the immediate in-frame transfer of all coding regions into virtually any protein expression vector. These transfers allow the addition of peptide tags to either or both end of the proteins. This repository, called the FLEXGene Repository (for Full-length Expression-ready), will enable the high-throughput (HT) screening of protein function for the entire set (or any customized subset) of genes using any method of *in vitro* or *in vivo* expression.

The most exciting part of this project has been the ease with which the clones from the repository can be rapidly incorporated in HT biological experimentation. Using automated gene transfer methods and protein purification it is now possible to purify thousands of proteins. Using HT retroviral methods, proteins capable of driving cell migration, altering the morphogenesis of normal epithelial structures, and affecting substrate dependent growth have been identified. A novel form of protein microarray, called nucleic acid programmable protein array (NAPPA), has been developed. This method substitutes the printing of proteins on the array with printing cDNAs encoding the proteins. Thus, the array is a DNA array that can be converted into a protein array by adding cell free protein synthesis machinery. This obviates the need to purify proteins, produces human proteins in a mammalian milieu, and avoids concerns about protein stability on the array because the proteins are made just-in-time for assay. NAPPA arrays can be used to study protein-protein interactions, protein-drug interactions and as tools to search for disease biomarkers.

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

“MELT: A Novel Method for RNA Preservation and Isolation from Solid Tissue”

Gary J. Latham, Ph.D., and Heidi J. Peltier

¹*Ambion, Inc., Austin, TX*

Intact RNA is an obligate starting material for expression profiling technologies such as real-time RT-PCR and microarrays that can link specific molecular events with disease phenotypes. The isolation of RNA from tissue currently requires invasive mechanical

force to disrupt the cellular architecture. Such mechanical approaches are cumbersome, low throughput, and risk biohazard exposure. During the phase I IMAT-funded period, we demonstrated the feasibility of a hands-off, closed tube tissue disruption method termed “MELT” (M ulti- E nzymatic L iquefaction of T issue).

MELT enlists potent catabolic enzymes to liquefy tissue within minutes without invasive mechanical force. High yields of intact RNA are obtained after enzymatic tissue digestion and RNA purification using a novel magnetic bead chemistry. A simple alteration in the protocol also allows the recovery of genomic DNA. RT-PCR and microarray expression profiling studies of MELT RNA reveal an excellent correlation with RNA extracted using popular methods. Importantly, MELT enzymes destroy cellular RNases and stabilize RNA in tissue lysates for more than a week at ambient temperatures. Additionally, MELT is compatible with many freshly harvested and flash-frozen tissues, and including tumor specimens.

In the current phase II funded period, we are working to further accelerate the rate of tissue digestion and maximize the quality of the isolated RNA, and link these advances with improved RNA-binding chemistries to enable more streamlined nucleic acid purification. These advances promise faster, simpler, safer, and more robust methods for stabilizing and quantifying gene expression in tissues through innovations in RNA stability, closed-tube tissue disruption, and rapid single-tube sample preparation.

Paul M. Lizardi, Ph.D.

Yale University School of Medicine

Analysis of Genetic and Epigenetic Alterations in DNA Archives Generated from Small Lesions and Tumors of the Head and Neck

Paul M. Lizardi, Ph.D.¹, José M. Lage Tao Huang², Andrew Dyer¹, Katie Crist¹, Rick Segraves⁴, Donna Albertson⁴, Diane Kowalski¹, Hongyu Zhao², Clarence Sasaki³, and Jose Costa¹

¹Department of Pathology, ²Department of Epidemiology and Public Health, and

³Department of Surgery, Yale University School of Medicine, New Haven, CT,

⁴Comprehensive Cancer Center, University of California, San Francisco, CA

Methods are now available which enable amplification of the entire human genome, starting from samples of a few hundred cells. We are using head and neck cancer as a model to establish and validate procedures for DNA archiving of neoplastic and pre-neoplastic lesions. Samples of head and neck malignancies are being collected prospectively. Archival DNA is stored prospectively before and after whole genome amplification. The amplified, archival DNA samples are being assayed for human papillomavirus (HPV) infection. The samples are additionally being processed for analysis of gene losses and gains, using BAC-array-CGH, which is being performed at UCSF.

A closely related activity, initiated with pilot study seed funding from the Yale Cancer

Center, is the analysis of unamplified DNA from the same tumor DNA archives, using a custom Nimblegen microarray designed to report changes in DNA methylation status. Microarrays have been designed which probe 340,000 loci across the human genome. Probed loci include CpG islands associated with known genes, other unique CpG islands, and CpG islands located within repetitive DNA elements. Proof-of-concept analysis reveals a large number of changes in DNA methylation levels relative to morphologically normal tissue. A substantial number of methylation changes occur at CpG islands associated with repetitive DNA. In the future, the information generated by these microarrays can be combined with the gene gain/loss information from BAC-array-CGH, to enable improved tumor class comparison, class discovery, and class prediction.

We will use a bioinformatics framework for analysis of the entire data set of genetic and epigenetic alterations generated from these studies. Computational and statistical tools will be used to construct classification schemes based on distance-based trees, as well as different clustering algorithms, utilizing the complete data set of array-CGH, DNA methylation, and HPV infection status observations. Our goal is to derive a conditional risk model for those patients that develop additional head and neck cancers in the future. Given a large enough data set derived from the prognostic follow-up sampling, we will make an effort to generate a multi-factorial model for risk prediction.

G. Mike Makrigiorgos, Ph.D.
Dana-Farber Cancer Institute

Genome Amplification Tolerant to Sample Degradation

G. Mike Makrigiorgos, Ph.D.¹, Gang Wang, Ph.D.¹, Jin Li¹, Elizabeth Maher, M.D., Ph.D.¹, and Max Loda, M.D.¹

¹Dana-Farber Cancer Institute, Boston, MA

Genomic, epigenetic, and gene expression analysis from archived formalin-fixed paraffin embedded (FFPE) prostate tissue samples with known clinical outcomes provides a unique opportunity for obtaining genetic information leading to improved prostate cancer diagnosis, prognosis and therapy. However, extensive genotyping or microarray profiling of genomic DNA and RNA derived from homogeneous cell populations within these samples often requires laser capture microdissection coupled to whole genome/RNA amplification prior to screening. Major hurdles to this process are the introduction of amplification bias, the inhibitory effects of formalin fixation on DNA amplification and the damage introduced by laser capture microdissection itself. Despite development of methods for unbiased whole genome amplification from intact DNA/RNA, the inability to amplify degraded nucleic acids in an unbiased fashion remains a problem.

We have developed RCA-RCA, a novel method based of isothermal rolling-circle amplification that overcomes the main limitations and promises to provide the needed link between obtaining a minute biopsy from partially degraded, FFPE samples and

genotyping or micro-array screening. Our published work (Genome Research, 14:2357, 2005) and preliminary data have validated RCA-RCA for use with FFPE samples of low or modest degradation. The planned work in this 'sample preparation' R21-R33 project will enable RCA-RCA to recover 'every bit' of nucleic acid that remains intact from minute biopsies and apply molecular assays to the amplified sample using valuable, highly degraded FFPE samples currently considered 'unusable'. It will also become possible to identify when samples should be discarded prior to investing resources in screening.

Deirdre R. Meldrum, Ph.D.

University of Washington

A Real-Time PCR Analyzer for the ACAPELLA-5K Capillary-Based Sample Processor

Deirdre R. Meldrum, Ph.D.¹, Mark R. Holl, Ph.D.¹ Patrick N. Ngatchou¹, Charles H. Fisher¹, Mohan S. Saini¹, Shawn K. McGuire¹, Jianchun Dong¹, Timothy T. H. Ren¹, Douglas A. Donaldson², David L. Cunningham² Stephen E. Moody², and William H. Pence²

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

Development and performance evaluation of a real-time PCR scanner add-on module for the ACAPELLA-5K automated, high-precision, fluid-handling system is presented. The laser-induced fluorescence scanner uses high-sensitivity photomultiplier tubes and appropriate spectral filters for the detection of three spectral wavelengths. PCR thermal cycles are performed using Peltier-effect thermoelectric elements that drive a slew rate of 4 ° C/s, and have setpoint tracking accuracy of ± 0.5 ° C.

Results are presented for the detection of t(14;18) gene, a translocation associated with the cancer follicular lymphoma, in RL-7 cells. As a measure of lower detection threshold in the presence of high background DNA, amplification of 2 copies of the t(14;18) gene is demonstrated in a human genomic DNA background of 40 ng/ μ L in 20% of capillaries tested. Minimum reaction volumes are demonstrated for the albumin assay down to 0.5 μ L. Hardware enhancements address precision positioning of the reaction volume in the capillary within the laser scan region, control of reaction evaporation during thermal cycling, and detailed plans for integration of the scanner module within the A5K instrument. A Neural network-based solution to the problem of estimating initial DNA concentration is presented. The neural network encoder is trained on features from amplification curves obtained after running hundreds of samples with known initial concentrations on a high-throughput fluorescence real-time PCR analyzer. The trained encoder is then used to estimate the initial concentration of unknown samples. Preliminary results show that this method is comparable to the conventional standard curve technique, with no assumption made about the underlying reaction.

Dobrin Nedelkov, Ph.D.

Intrinsic Bioprobes, Inc.

Investigating Protein Diversity in Human Populations

Dobrin Nedelkov, Ph.D.¹, Urban A. Kiernan¹, Eric E. Niederkofler¹, Kemmons A. Tubbs¹, and Randall W. Nelson¹

¹*Intrinsic Bioprobes, Inc., Tempe, AZ*

Plasma proteins represent an important part of the human proteome. While recent proteomics research efforts focus largely on determining the overall number of proteins circulating in plasma, it is equally important to delineate protein variations among individuals, as they can signal the onset of diseases and be used as biological markers in diagnostics.

To date there has been no systematic proteomics effort to characterize the breadth of structural modifications in individual proteins in the general population. As part of this IMAT Phase II project we have undertaken a Population Proteomics study to define gene- and protein-level diversity that is encountered in the general population. Twenty-five plasma proteins from a cohort of ninety-six healthy individuals were investigated via affinity-based mass spectrometric assays. A total of 76 structural forms/variants were observed for the 25 proteins within the samples cohort. Posttranslational modifications were detected in 18 proteins, and point mutations were observed in 4 proteins. The frequency of occurrence of these variations was wide-ranged, with some modifications being observed in only one sample, and others detected in all 96 samples.

Even though a relatively small cohort of individuals was investigated, the results from this study illustrate the extent of protein diversity in the human population, and can be of immediate aid in clinical proteomics/biomarker studies by laying a basal-level statistical foundation from which protein diversity relating to disease can be evaluated.

Tsukasa Oyama

University of Texas Southwestern Medical Center

Development of a High Throughput Diagnostic Assay for Lung Cancer

No abstract available

Lewis K. Pannell, Ph.D.

University of South Alabama

Automated Glycoanalysis of Cancer-Related Proteins

Lewis K. Pannell, Ph.D.¹, T. R. Hubler¹, K. T. Krulla¹, R. A. Torres¹, F. Mbeunkui¹, J. C. Ruiz¹, L. A. Shevde¹, and R. S. Samant¹

¹*Proteomics and Mass Spectrometry Facility, Cancer Research Institute, University of South Alabama, Mobile, AL*

Many cell surface and secreted proteins are glycosylated. Changes in glycosylation are

associated with various aspects of cancer progression including cell-cell adhesion, immune surveillance and metastasis. Mass spectrometry is a powerful tool for the analysis of post-translational modifications (PTMs) such as glycosylation due to its ability to detect changes in peptide mass that correspond to modifications. We have previously developed automated software to simplify the identification of protein glycosylation.

Our focus is the characterization of glycosylation sites on cell surface and secreted proteins that are implicated in cancer. While immunoprecipitation (IP) followed by Western blot analysis is a standard procedure, preparation of protein samples using conditions suitable for mass spectrometry requires considerable modification. Proteins are being isolated by IP using an antibody coupled to either an amine-reactive resin or Protein A/G agarose beads, and samples are digested either directly from the resins or after elution using a MS-friendly method that minimizes antibody contamination.

Secreted proteins are being isolated from an optimized “secretory proteome” fraction. Thus, Galectin-3 Binding Protein (Galectin-3BP) was immunoprecipitated from conditioned media, detected by mass spectrometry using an in-gel tryptic digest, and is now identified after a direct digest of the IP. Cell surface glycoproteins present additional complications, but our method is being adapted to address these problems.

In parallel with the isolation strategies, the automated software is being enhanced to assist in the characterization of glycosylation on the immunoprecipitated proteins, especially interpretation in the presence of contaminating or co-isolating proteins.

Ramila Philip, Ph.D.
Immunotope, Inc.

Identification of Auto Antigens in Ovarian Cancer

Ramila Philip, Ph.D.¹, Sid Murthy¹, Jonathan Krakover¹, Beth Vertin¹, and Kim Lyerly²

¹Immunotope, Inc., Doylestown, PA, ² Duke University, Durham, NC

The overall goal of the project is to conduct proof-of-concept studies to identify tumor associated antigens (TAA) that will lead to the development of i) early stage diagnostics and ii) immunotherapeutics that induce strong cellular (T cell) and humoral (B cell) responses against ovarian tumors.

Proteomic analysis to identify tumor-associated antigens reactive to serum immunoglobulin from primary ovarian cancer patients at different stages of disease, with the goal of identifying antigens common to the majority of patients sampled. This is achieved by immunoprecipitation of TAA from primary tumor lysates using patient serum as the source of auto antibodies and serum from healthy donors as controls to identify auto antibodies found only in patient serum. The TAAs are characterized by mass spectroscopy and a TAA proteomics database is generated for comparative analysis. The TAAs are further analyzed to identify the epitopes specific for the auto

antibodies in cancer patient serum. Since native conformation and post-translational processing can have profound effects on antibody recognition, we further characterize glycosylation patterns on these antigens and determine whether they play a role in antibody recognition.

First set of experiments have resulted in a number of putative targets reactive to autoantibodies in patients' serum. Additional patients and healthy serum sample analysis are under way to confirm the data and to develop an early detection diagnostics for ovarian cancer.

Leslie B. Poole, Ph.D.

Wake Forest University School of Medicine

Elucidation of Redox Signaling Networks Through Cysteine Sulfenic Acid Detection in Peroxide-Responsive Proteins

Leslie B. Poole, Ph.D.¹, S. Bruce King², Jacquelyn S. Fetrow³, and Larry W. Daniel¹

¹Department of Biochemistry, Wake Forest University School of Medicine, ²Department of Chemistry, ³Departments of Physics and Computer Science, Wake Forest University, Winston-Salem, NC

Redox regulation of enzymes and transcription factors is widely recognized to play a critical role in receptor-mediated signal transduction processes. A number of cell stimuli are now known to operate not only through phosphorylation cascades but also through hydrogen peroxide bursts generated by mitogen-activated NADPH oxidases. Redox-sensitive metal and/or cysteinyl centers in responsive proteins are then likely to elicit functional changes through peroxide-mediated oxidation, although much remains to be discovered as to which proteins undergo these changes.

Because the first metastable product of cysteine oxidation is cysteine sulfenic acid (Cys-SOH), this species represents an excellent target for discovering and identifying peroxide-modifiable cysteine residues in cellular proteins. While several methods have allowed for the detection of Cys-SOH formation at catalytic and regulatory sites in purified proteins, methods for the demonstration of cysteine oxidation *in vivo* have lagged far behind. Using the unique reactivity of the reagent dimedone toward Cys-SOH, our laboratories have been developing methodology for identifying Cys-SOH formed in proteins *in vivo*. We have recently generated several R-SOH-reactive compounds that specifically incorporate a fluorescent label into a Cys-SOH-containing model protein. Use of these reagents to trap and label Cys-SOH in proteins *in situ* or rapidly at the time of cell disruption will also yield the opportunity to discover hydrogen peroxide-modifiable proteins in a whole-cell, proteomics format.

Such proteomics experiments will allow us to elucidate the role of redox regulation in signal transduction and pave the way toward a molecular understanding of the interactions between the many signal transduction networks in the cell.

Supported in part by R21 CA112145 to the authors (L.B.P., PI), and by an Established

Investigatorship from AHA to L.B.P (0140175N).

Suzie H. Pun, Ph.D.

University of Washington

Nanoparticles for Efficient Delivery to Solid Tumors

Suzie H. Pun, Ph.D.¹, and T. Tyrel Goodman¹

¹*Department of Bioengineering, University of Washington, Seattle, WA*

New methods for *in situ*, molecular analysis of cancer include smart imaging agents that recognize tumor-specific proteins, molecular beacons that detect specific nucleic acid sequences, and nucleic acid therapeutics that alter the protein expression profiles of tumor cells. Application of these technologies for effective identification and treatment of metastatic cancer requires material amenable to systemic administration.

Nanoparticle formulations of these agents offer protection and stabilization *in vivo* and mediate concentrated delivery of material to tumor cells by selective extravasation through leaky tumor vessels. However, a major limitation of nanoparticles for tumor delivery is restricted transport through interstitial tumor due to their size and to opposing interstitial pressure.

The major goal of this project is to develop a non-invasive approach to overcome transport barriers that prevent nanoparticle penetration of solid tumors. We propose to mimic mechanisms utilized by bacterial pathogens to efficiently invade tissues. Our specific aims are to modify nanoparticles with bacteria-derived proteins and to demonstrate improved delivery efficiency in cultured cells. If developed, this technology may eventually be applied *in vivo* to deliver agents for the molecular analysis of metastatic tumors with high specificity and efficiency.

Arfaan Rampersaud, Ph.D.

Columbus NanoWorks, LLC

Nanobeads for Cancer Cell Selection

Arfaan Rampersaud, Ph.D.¹, Kristie Melnik², Rob Walczak¹ and Jeffrey Chalmers²

¹*Columbus NanoWorks, LLC, Columbus, OH, 2*Department of Chemical Engineering, The Ohio State University, Columbus, OH

The aim of this project is to develop high-resolution magnetic nanoparticles for use in Quadrupole Magnetic Sorting (QMS). QMS is a flow-through immunomagnetic separation system that can provide sensitive enrichment of circulating cancer cells in blood as well as other biological fluids. Optimal cellular separation by QMS requires immunomagnetic particles having high magnetic susceptibility, narrow particle size distribution, and high-density attachment sites for antibodies. Commercial immunomagnetic beads are either too large, lack size uniformity, or have low magnetic susceptibility.

In Specific Aim 1 we will synthesize and physically characterize monodisperse paramagnetic nanoparticles made by Columbus NanoWorks. The resulting particles will be analyzed for their particle field interaction parameter values which is a major determinant of the effectiveness of magnetic nanoparticles in cellular separation. These nanoparticles will possess a narrow size distribution (between 30 to 50 nm) and a field interaction parameter at least 10 times higher ($2-8 \times 10^{-24} \text{ m}^3$) than the Miltenyi MACS reagents. We will use different attachment approaches to create high-density attachment sites for antibodies and streptavidin at least 200 to 300 ng/cm². Magnetic nanobeads need to have low particle agglomeration due to protein-protein interactions, show at least 90% monodispersity, and have high affinity binding to target molecules as determined by CTV analysis. Finally, we will evaluate our magnetic nanoparticles for immunomagnetic detection and separation of ovarian tumor cells by QMS. Demonstrating the detection of 1 tumor cell a population of 10^7 whole blood cells will be taken as the proof of principal for this project.

Bing Ren, Ph.D.

University of California San Diego School of Medicine

Mapping Transcription Factor Binding Sites in the Human Genome by Genome-wide Location Analysis

Bing Ren, Ph.D.^{1,4}, Tae Hoon Kim¹, Leah O. Barrera¹, Ming Zheng², Chunxu Qu¹, Michael A. Singer³, Todd A. Richmond³, Yingnian Wu³, and Roland D. Green³

¹Ludwig Institute for Cancer Research, La Jolla, CA, ²Department of Statistics, University of California, Los Angeles, CA, ³Nimblegen Systems, Inc., Madison, WI, ⁴Department of Cellular and Molecular Medicine and Moores Cancer Center, University of California, San Diego, School of Medicine, La Jolla, CA

A large number of transcription factors have been implicated in tumorigenesis, but our understanding of the mechanisms by which these factors contribute to cancer is still limited by a lack of comprehensive knowledge of their target genes in the cancer cells. The objective of our project is to develop a genome wide location analysis (GWLA) technique that allows the rapid identification of genomic binding sites for transcription factors in the human genome. Our approach involves formaldehyde fixation of cells, immunoprecipitation of crosslinked chromatin DNA fragments, and detection of enriched transcription factor binding sites with DNA microarray technologies.

The effectiveness of this approach is demonstrated by systematic mapping of the sites of RNA polymerase II preinitiation complex (PIC) binding throughout the human genome. A series of high-density oligonucleotide arrays were fabricated to represent the non-repetitive DNA of the human genome and used for hybridization. Computational algorithms were developed to process the DNA microarray data and determine the PIC binding sites to within 100bp resolution. Multiple assays were performed to verify the high accuracy and specificity of the results. The resulting map of PIC binding defined 10,567 active promoters corresponding to 6,763 known genes and at least 1,196 un-annotated transcriptional units. This map suggested extensive use of multiple promoters

by the human genes and widespread clustering of active promoters in the genome, and provided a global view of the functional relationships among transcriptional machinery, chromatin structure and gene expression in human cells.

The technology that we developed here should be useful for studying the functions of transcription factors associated with various human cancers. In the next phase, we plan to use this technology to systematically examine the function of several oncogenic transcription factors, including c-Myc and b-catenin, in human tumors.

Beerelli Seshi, M.D.

David Geffen School of Medicine at UCLA

A Non-Gel-Based Approach to Mapping the Proteome of the Human Bone Marrow Stromal Cell

Beerelli Seshi, M.D.¹, and Guadalupe Salazar¹

¹Department of Pathology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, David Geffen School of Medicine at UCLA, Los Angeles, CA

The bone marrow (BM) stromal mesenchymal stem or progenitor cell (MPC), relatively well recognized for its developmental plasticity, represents the chief component of the BM microenvironment and is equally well-known for its ability to support hematopoiesis by promoting growth and development of normal hematopoietic cells as well as leukemia cells. However, the molecular foundation of its functions is less well understood.

We report differential protein expression profiling of leukemia-associated MPC using a recently available non-gel based, isobaric amine-reactive tagging reagent (iTRAQ) technology, which allows simultaneous quantitative protein expression measurement of up to four samples multiplexed prior to LC/MS/MS. MPCs were culture-expanded from BM samples obtained from two normal individuals and two patients with a hematopoietic malignancy (one with acute lymphocytic leukemia and the other with multiple myeloma). Because culture-expanded stromal cells contain a significant percent of macrophages and hematopoietic cells, we first documented the proteomic changes in purified vs. unfractionated normal stromal cells, validating the purification methodology.

Next, we compared the proteomic changes in leukemia-associated vs. normal purified stromal cells. Focusing on three main aspects of the analysis, we obtained the following results: a) Primary data analysis resulted in identification and quantitative determination of a total of 504 distinct proteins detected in either the purified or unfractionated normal stromal cells and a total of 905 distinct proteins detected in either the leukemia-associated or normal purified stromal cells with >95% confidence which, to our knowledge, represents a largest number of proteins identified using LC/MS/MS involving iTRAQ technology. b) Comparative protein expression analysis resulted in identification of 73 proteins that were differentially expressed in leukemia-associated stromal cells in reference to normal stromal cells. c) Classification of identified stromal

proteins using gene ontology provided insight into the biological functionality of the proteins under study. The identified stromal proteins were over-represented in 33 biological pathways, under-represented in 10 and not represented at all in 17 out of 60 pathways examined.

The study further identified a variety of multilineage mesenchymal (e.g., muscle cell, bone cell, fibroblast) and other lineage (neural cell) marker proteins, indicative of stromal cell's potential multilineage differentiation capacity. In summary, the present study remarkably identified over 900 proteins, presenting the first picture of the human bone marrow stromal cell proteome. On the basis of this success and combined with appropriate pre-fractionation of the sample proteins into sub-proteomes, we expect to be able to undertake a global analysis of many thousands of stromal cell proteins with the objective of determining their role in normal and leukemic hematopoiesis.

Shan-Rong Shi, M.D.

University of Southern California Keck School of Medicine

Protein-Embedding Technique: A Potential Approach to Standardization of Immunohistochemistry for Formalin-Fixed, Paraffin-Embedded Tissue Sections

Shan-Rong Shi, M.D.¹, Cheng Liu¹, Jeanette Perez¹, and Clive R. Taylor, M.D., Ph.D.¹
¹Department of Pathology, University of Southern California, Keck School of Medicine, Los Angeles, CA

A serial study was carried out to develop a protein-embedding technique for standardization of immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) tissue sections. The key issue is to find an optimal matrix material as a protein-carrier. This matrix must have two phases, a liquid phase to allow uniform mixing of a protein, and a solid phase forming a 'block' that can be fixed and processed in the same manner as human tissue. The standard protein block would serve as a source of thin sections for control of IHC, and therefore must also withstand the boiling treatment of antigen retrieval (AR). Several methods have been tested such as protein absorption method, a method of direct mixing protein into matrix media using a variety of natural as well as polymer-based materials.

After careful experiments, a method was developed utilizing polymer microsphere (beads) as a support medium for protein. The resulting suspension of protein coated beads can then be fixed, embedded in paraffin, sectioned and applied as the standard reference material for routine IHC of FFPE tissue. Several proteins have been coated successfully onto the surface of the beads and have then been visualized as positive surface markers upon microscopy. The method showed particular promise for quantitative IHC of Her-2/neu, currently a major issue in clinical and research of breast cancer. The utility and limitations of AR can also be assessed by this method. In development of standard reference materials for IHC, we recognized that a cooperative research team including experts of chemical engineering, computer science, biochemistry and IHC is critical for accomplishing our specific aims.

In October of 2003, we started to establish such a cooperative team with the Peking University and Tsinghua University based on our specific aims focusing on standardization of IHC. Further study is ongoing to verify various concentrations of protein coated on the surface of matrix to create a potential reference material that may be used to quantitative IHC.

Ie-Ming Shih, M.D., Ph.D.
Johns Hopkins Hospital

Inactivation of the MAPK Pathway as a Potential Target-based Therapy in Ovarian Serous Tumors with KRAS or BRAF Mutations

Ie-Ming Shih, M.D., Ph.D.¹, Gudrun Pohl¹, Chung-Liang Ho¹, Robert J. Kurman¹, Robert Bristow¹, and Tian-Li Wang¹

¹*Department of Pathology, Johns Hopkins Hospital, Baltimore, MD*

Activation of mitogen-activated protein kinase (MAPK) occurs in response to various growth stimulating signals and as a result of activating mutations of the upstream regulators, KRAS and BRAF, which can be found in many types of human cancer. To investigate the roles of MAPK activation in tumors harboring KRAS or BRAF mutations, we inactivated MAPK in ovarian tumor cells using CI-1040, a compound that selectively inhibits MEK, an upstream regulator of MAPK and thus prevents MAPK activation. Profound growth inhibition and apoptosis were observed in CI-1040 treated tumor cells with mutations in either KRAS or BRAF in comparison to the ovarian cancer cells containing wild-type sequences. Long serial analysis of gene expression (SAGE) identified several differentially expressed genes in CI-1040 treated MPSC1 cells harboring an activating mutation in BRAF (V599L).

The most striking changes were downregulation of cyclin D1, COBRA1 and transglutaminase-2 and upregulation of TRAIL, thrombospondin-1, optineurin and palladin. These patterns of gene expression were validated in other CI-1040 treated tumor cells based on quantitative PCR. Constitutive expression of cyclin D1 partially reversed the growth inhibitory effect of CI-1040 in MPSC1 cells. Our findings indicate that an activated MAPK pathway is critical in tumor growth and survival of ovarian tumors with KRAS or BRAF mutations and suggest that the CI-1040 induced phenotypes depend on the mutational status of KRAS and BRAF in ovarian tumors.

Robert H. Singer, Ph.D.
Albert Einstein College of Medicine

Gene Expression Profiling in Single Cells within Tissue

Robert H. Singer, Ph.D.^{1,3}, Paola Capodiecì¹, Michael Donovan¹, Heidi Buchinsky¹, Yusuf Jeffers¹, Carlos Cordon-Cardo², William Gerald², Jon Edelson¹, Shailesh M. Shenoy³, Melissa Lopez-Jones³, and Rossanna Pezo³

¹*Aureon Laboratories, Yonkers, NY, ²Memorial Sloan-Kettering Cancer Center, New*

York, NY, ³Albert Einstein College of Medicine, Bronx, NY

We have developed a robust fluorescent *in situ* hybridization (FISH) technique for identifying multiple, unique nascent RNA transcripts in single nuclei that permits the detection of multiplex gene expression patterns in single cells within archival formalin-fixed, paraffin-embedded histological sections. This allows a tissue based quantitative analysis of gene expression patterns to be used as predictive signatures at microanatomical detail. This integration of histological and cellular features with molecular profiles will significantly enhance the understanding how cells respond to their microenvironment and has the potential for elucidating basic biological mechanisms of disease processes. This approach could also improve patient management by assisting in early diagnosis and objective patient prognosis, including outcome and response to treatment. As an example, we have investigated the gene expression signatures in prostate tumor and normal cells from patients, and in mouse colon.

Antoine M. Snijders

University of California at San Francisco

Rare Amplicons Implicate Frequent Deregulation of Cell Fate Specification Pathways in Oral Squamous Cell Carcinoma

Antoine M. Snijders¹, Brian L. Schmidt², Jane Fridlyand³, Nusi Dekker⁴, Daniel Pinkel⁵, Richard C. K. Jordan^{4,6}, and Donna G. Albertson, Ph.D.^{1,5}

¹Cancer Research Institute, ²Department of Oral and Maxillofacial Surgery, ³Department of Epidemiology and Biostatistics, ⁴Department of Stomatology, ⁵Department of Laboratory Medicine, ⁶Department of Pathology, University of California at San Francisco, San Francisco, CA

Genomes of solid tumors are characterized by gains and losses of regions, which may contribute to tumorigenesis by altering gene expression. Often aberrations encompass whole chromosome arms, which makes identification of candidate genes in these regions difficult. Here, we focused on narrow regions of gene amplification to facilitate identification of genetic pathways important in oral squamous cell carcinoma (SCC) development.

Genome-wide analysis of copy number aberrations in 89 oral SCC taken from four different sites in the oral cavity found a number of frequent low level gains and losses and 18 regions of recurrent amplification. Hierarchical clustering of the array CGH data revealed two main branches in the dendrogram, one of which is significantly enriched with tumors with mutations in TP53 exons 5-8 (Fisher exact test p-value = 0.001). Regardless, of position in the dendrogram, low level copy number alterations significantly associated with TP53 mutational status after maxT correction for multiple testing included -8p, +distal 8q, -10q, -11q and -18q. In addition, mutation of TP53 was positively correlated with amplification of CCND1 (Fisher exact test p-value = 0.009), and EGFR (Fisher exact test p-value = 0.036).

We used expression analysis to identify candidate driver genes for nine amplicons with

boundaries spanning less than 3 Mb. We found genes involved in integrin signaling (TLN1), survival (YAP1 , BIRC2), and adhesion and migration (TLN1 , LAMA3 , MMP7), as well as members of the hedgehog (GLI2) and notch (JAG1 , RBPSUH , FJX1) pathways to be amplified and over-expressed. Deregulation of these and other members of the hedgehog and notch pathways implicates deregulation of developmental and differentiation pathways, cell fate misspecification, in oral SCC development.

Steven A. Soper, Ph.D.
Louisiana State University

Flow-Through Lab-on-a-Chip Biosensors for the Collection of Rare Circulating Tumor Cells and the Analysis of Their Intracellular Contents

Steven A. Soper, Ph.D.¹, Robin L. McCarley¹, Michael C. Murphy¹, David Spivak¹, Feng Xu¹, Andre Adams¹, Suying Wei¹, Subramanian Balamurugan¹, Jost Gottert¹, Proyag Datta¹, and Matseuz L. Hupert¹

¹Center for BioModular Multi-Scale Systems, ²Center for Advanced Microstructures and Devices, Louisiana State University Baton Rouge, LA

We are developing polymer-based, modular microfluidic systems that can be produced in a high production mode at low costs using replication micro- and nanotechnologies and assembled into three-dimensional architectures to carry out complex multi-step bioassays. The fabrication methods make the integrated systems appropriate for one-time use, which is attractive for clinical applications. In this work, a microsampling unit is being constructed that can sample macro-volumes of fluids, search for low frequency events (circulating tumor cells) and concentrate them to nanoliter volumes.

The sampling unit consists of a series of channels (20) that are narrow and deep (20 μm x 250 μm). Using a linear flow velocity of ~ 1 cm/s, we can process 1 mL of a clinical sample in less than 10 min and concentrate the target material to a volume of 190 nL. The target cells we are interested in capturing over-express EpCAM (integral membrane protein). The target cells are captured using molecular recognition elements, which in this case are aptamers that are assembled onto the walls of the microfluidic device using self-assembled monolayers (SAMs). We can capture nearly 100% of circulating tumor cells when the device is configured appropriately (channel width similar to the cell diameter).

In addition, microfluidic modules are being developed that permit the electrodynamic lysis of the captured cells, accumulation of certain intracellular materials (proteins or nucleic acids) and the molecular identification of certain biomarkers released from the lysed cells using hybrid microarrays configured onto planar polymer waveguides.

Clive R. Taylor, M.D., Ph.D.
University of Southern California, Keck School of Medicine

Flmmunohistochemical Evaluation of Histoids (Haux Tissue) by Using Further

Diluted Primary Antibodies: A “Critical Point” Strategy

Clive R. Taylor, M.D., Ph.D.¹, Shan-Rong Shi¹, Cheng Liu¹, Jeanette Perez¹, Marylou Ingram², and S. Ashraf Imam²

¹Department of Pathology, University of Southern California, Keck School of Medicine, Los Angeles, CA, ²Huntington Medical Research Institute, Pasadena, CA

This faux tissue of “histoids” shows breast cancer cells invading into the stroma consisted of fibroblasts, behaving as a tissue of invasive breast cancer. Thus, the “histoids” can be processed and analyzed in the same way as that of human tissue sample serving as a “reference standard.” However, multiple batches of “histoids” are necessary in order to achieve a required size of cell block for routine process of tissue. Therefore, to assure the consistency of protein expression among these multiple batches of “histoids” is a critical issue which must be addressed. How to evaluate the degree of consistency of protein expression in different batches of cell culture sample is a new topic for our research project of histoids. To our knowledge, there have not been articles documented about this issue based on online search. Although a few investigators found variable gene expression in cell lines that had been passed many generations, no one documented a method that can be adopted to measure slight alterations of protein expression among serial batches of cultured cell samples.

Most recently, we tried to further dilute five primary antibodies to reach a “critical point” that is one step back from negative IHC staining. These highly diluted primary antibodies (critical points of 5 markers are: Topo II 1:25,000, HER-2 1:500, DO7 1:500,000, vimentin 1:100, and CK7 1:15,000 based on regular working solution of each antibody). We have tested 20 samples of histoids collected in 2004. Our preliminary work indicated slight variable protein expressions of some proteins (antigens) existing among some batches. It is expected to establish optimal reference standard including the “histoids” approach for standardization of routine IHC.

In addition, microfluidic modules are being developed that permit the electrodynamic lysis of the captured cells, accumulation of certain intracellular materials (proteins or nucleic acids) and the molecular identification of certain biomarkers released from the lysed cells using hybrid microarrays configured onto planar polymer waveguides.

Paul Tempst, Ph.D.
Memorial Sloan-Kettering Cancer Center
Tumor-Specific Serum Peptidome Patterns

*Paul Tempst, Ph.D.¹, Josep Villanueva¹, Martin Fleisher¹, and Richard Robbins (Co-PI)¹
¹Memorial Sloan-Kettering Cancer Center, New York, NY*

Recent studies have established distinctive serum polypeptide patterns through mass spectrometry that may correlate with clinically relevant outcomes. Wider acceptance of these 'signatures' as valid biomarkers for disease may follow sequence characterization

of the components and elucidation of the mechanisms by which they are generated.

To this end, we have developed an automated platform for simultaneous measurement of peptides in serum using magnetic, reversed-phase beads for analyte capture and a MALDI-TOF MS read-out. This system is more sensitive than surface capture on chips as spherical particles have larger combined surface areas than small-diameter spots. Coupled to high-resolution MS and MS/MS, hundreds of peptides can be detected in a droplet of serum. Automation facilitates throughput and ensures reproducibility. We also developed a minimal entropy-based algorithm that simplifies and improves alignment of spectra and subsequent statistical analysis.

Using our system, we show that a limited subset of serum peptides provides accurate class discrimination between patients with three types of solid tumors and controls. Sequence analysis revealed that these peptides fall into several clusters, and that most are generated by amino-peptidase activities that confer 'cancer-type'-specific differences superimposed on the proteolytic events of the ex-vivo coagulation and complement degradation pathways. This small but robust set of marker peptides provides highly accurate class prediction for an external validation set of prostate cancer samples. These findings have important implications for future biomarker discovery efforts. We will apply our technology to assess if serum peptide signatures can identify occult metastasis in a large group of thyroid cancer survivors.

Derek Thirstrup
Engineering-Arts

Piezoelectric Pipetting Technology for DNA Analysis

Derek Thirstrup¹, Peter Wiktor¹, Jim McGill¹, Ben Warrick¹, Rob Sullivan², and Helmut Zarbl²

¹Engineering-Arts, ²Fred Hutchinson Cancer Research Center, Seattle, WA

Our research follows two distinct yet complementary paths. One path involves developing and testing novel piezoelectric pipetting, dispensing, sensing and housing technology and integrating this into a fully automated piezoelectric pipetting system. The other path involves cancer-related genomics research in collaboration with the Fred Hutchinson Cancer Research Center (F.H.C.R.C.).

The ultimate goal of the proposed research is to have the two paths come together, resulting in an automated, fully functional, and general-purpose piezoelectric fluid pipetting system with the reliability and performance to empower cancer related, genomic and functional genomics research. We have demonstrated the feasibility of using our piezoelectric pipettes in application to eGFP expression cell microarrays. Furthermore, we have collaborated with another company to develop piezoelectric pipetting technology to construct whole genome expression arrays and sell them commercially. We are currently developing novel, high-density shRNA microarrays. These loss-of-function arrays should allow for the screening of cellular function and will

make it possible to assay phenotypic silencing effects of specific genes on a genome-wide scale.

Our current work has also focused on controlling cell adhesion over the arrayed spots. We have data showing controlled cell adhesion over individual spots and regions devoid of cells in between adjacent spots.

P.O. Ts'o, Ph.D.

CCC Diagnostics, LLC

Personalized Anticancer Therapy (PAC) for Metastatic Breast Cancer Patients as a Model for Targeted Therapy

P.O. Ts'o, Ph.D.¹, S. Deamond¹, Z.P. Lum¹, S. Lesko¹, K. H. Tkaczuk², N. S. Tait², F. Feldman², and D. A. Van Echo²

¹CCC Diagnostics, Baltimore, MD, ²University of Maryland, Baltimore, MD

Development of PAC is based on: (1) a source of tumor cells/tissues from individual patients; (2) quantitative measurement of drug response indicators (DRI) in these tumor cells; (3) quantitative correlation between the expression of DRI and response to a specific drug. Circulating cancer cells (CCC) constitute a source of tumor cells, which represent metastatic secondary tumors. Blood samples were drawn from 90 breast cancer patients (289 samples). CCC's were detected in 88% of Stage IV patients (1 to 1283/ sample). Forty-four percent of Stage II and III patients had at least one blood sample positive for CCC.

HER-2/neu status was quantitatively assessed in CCC's via specific monoclonal antibody staining and computerized fluorescence microscopy analysis. In ongoing studies, HER-2/neu expression in patients with 5 or more CCC's was 80% concordant with the primary tumor.

An *in vitro* indexing system was established to correlate the cytotoxic effect of each drug to the corresponding DRI measurements. Significant correlations were evidenced by Spearman Rank coefficients of (+1) for HER-2/neu expression / Herceptin and (-.995) for estrogen receptor expression / Tamoxifen. The expression of various DRI in circulating cancer cells may be statistically indexed in this system to predict individual patients response to a related therapy.

Binghe Wang, Ph.D.

Georgia State University

Artificial Lectins: Boronolactins and Fluorescent Boronolactins

Binghe Wang, Ph.D.¹, Na Lin¹, Xingming Gao¹, Zhen Huang¹, Craig Altier², Lynette Johnston², Nicolas Carrasco¹, Wenqian Yang¹, Yanling Zhang¹, and Jun Yan¹

¹Department of Chemistry and Center for Biotechnology and Drug Design, Georgia

State University, Atlanta, GA, ²College of Veterinary Medicine, North Carolina State University, Raleigh, NC

Lectins are naturally occurring carbohydrate-binding proteins and are considered very important analytical tools in studying the roles and functions of various glycosylations. In addition, lectin binding to certain carbohydrate biomarkers has been known to trigger biological events such as apoptosis.

However, several factors have limited the application of lectins. First, there are only a limited number of lectins available, making it hard to achieve complete differentiation of various oligosaccharide structures in analyzing glycosylation patterns.

Second, there is little that can be done to tune the selectivity of existing naturally occurring lectins.

Third, lectins are proteins, which limit their application *in vivo*. In this presentation, we will discuss our effort in preparing designer lectin mimetics (boronolactins), which change fluorescent properties upon saccharide binding and can be “tuned” to recognize different carbohydrates.

Yue Wang, Ph.D.

Virginia Polytechnic Institute and State University

Diagnostic Classification and Clustering Using Genomic Data and Machine Learning

Yue Wang, Ph.D.¹, Yitan Zhu¹, Zuyi Wang², Jianhua Xuan³, Yibin Dong¹, Yuanjian Feng¹, and Robert Clarke⁴

¹Virginia Polytechnic Institute and State University, ²Children's National Medical Center, ³The Catholic University of America, ⁴Georgetown University Lombardi Cancer Center

The challenge of cancer treatment has been to target specific therapies to pathogenetically distinct cancer types or subtypes, to maximize efficacy and minimize toxicity. However, cancers with similar histopathological appearance can follow significant different clinical courses and show different responses to therapy. The recent development of gene microarrays provides an opportunity to take a genome-wide approach to predict clinical heterogeneity in cancer treatment and potentially discover new diagnostic and therapeutic targets.

This presentation will first report our exploratory work on developing a weighted Fisher regularization of multilayer perceptrons classifiers for multiclass diagnostic classification. Various optimization issues (e.g., input gene subset selection, classifier architecture design, model parameter initialization) will be addressed with the purpose of reducing the likelihood of local minima and easing the curse of dimensionality. The discussion will then focus on sample/gene clustering by VISDA-caBIG TM, where extensive comparisons with bottom-up hierarchical clustering and non-model based

competitive self-organizing map will be presented, and further considerations on unsupervised discriminatory yet compact gene subset selection will be proposed.

Virgil L. Woods, Jr., M.D.

University of California

Facile Characterization of Protein Structure/Function with Enhanced Peptide Amide Hydrogen/Deuterium Exchange Mass Spectrometry (DXMS)

No abstract available

Daniel G. Wright, M.D.

Boston University Medical Center

Effective Delivery of Antisense Peptide Nucleic Acid Oligomers into Cells by Anthrax 'Protective Antigen'

Daniel G. Wright, M.D.¹, Ying Zhang, Ph.D.¹, and John R. Murphy, Ph.D.¹

¹*Section of Molecular Medicine, Department of Medicine, Boston University Medical Center Boston, MA*

A detailed understanding of the genetic basis of neoplastic diseases has emerged during recent decades, encouraging efforts to develop genetically targeted reagents as both experimental tools and novel anticancer therapeutics. Peptide nucleic acid (PNA), a DNA mimic in which the phosphate deoxyribose backbone of DNA has been replaced by a pseudopeptide polymer, first described in 1991, has attracted particular interest as a gene-targeting reagent, since it is highly stable and binds to complementary RNA and DNA with high affinity and specificity.

However, because PNA oligomers resist cellular uptake, their development as tools for modifying gene expression in whole animal studies or as potential therapeutic agents has been limited. To explore the possibility that the transmembrane "transport" domains of microbial toxin proteins might serve as vehicles for cellular delivery of PNA, we have studied the ability of recombinant Anthrax "protective antigen" (APA), the non-toxic component of Anthrax toxin that mediates cell binding and delivery, to transport antisense PNA oligomers effectively into cells.

For our studies, we first generated CHO-K1 cell lines (CHO-Luc654) that had been engineered by stable transfection to express a modified luciferase gene (Luc-betaIVS2-654) interrupted by a mutant beta-globin intron-2 with an aberrant splice site that could be blocked by antisense PNA, thereby inducing luciferase expression as an indicator of antisense activity (ref. Sazani & Kole, J Clin Invest 112:481, 2003). We then synthesized PNA oligomers with poly-lysine tails and 18-mer nucleobase sequences antisense to a region of Luc-betaIVS2-654 pre-mRNA flanking the aberrant IVS2-654 splice site. As anticipated from prior reports (Nucleic Acids Res 29:3965, 2001), antisense PNA-(Lys)₈ oligomers demonstrated detectable sequence-specific activity in inducing luciferase expression in CHO-Luc654 cells when incubated with the cells for 48-72 hrs at micromolar concentrations. However, this activity was greatly amplified

when CHO-Luc654 cells were incubated with antisense PNA-(Lys)₈ together with APA (0.3-1.0 microgm/mL), which had no activity by itself, such that antisense activity could be detected at PNA concentrations as low as 30 nM. Antisense PNA-(Lys)₈ (300 nM), with but not without APA (0.3 microgm/mL), was also found by rtPCR to induce correctly spliced beta-globin transcripts in cultured erythroid progenitor cells obtained from a patient with beta-thalassemia intermedia (genotype, IVS2-654(betaC/betaE), kindly provided to us by Dr. Edmond Ma, Queen Mary Hospital, Univ. of Hong Kong). These studies provide proof-of-principle evidence that the transmembrane transport functions of microbial toxins (e.g. APA) can be harnessed to deliver antisense PNA oligomers effectively into cells. Since microbial toxin proteins can be molecularly engineered to achieve cell selective binding, as exemplified by the IL2-diphtheria fusion toxin, ONTAK(r), our findings support the possibility that novel anti-cancer agents, based on PNA oligomer constructs and modified microbial toxin proteins, can be developed that combine both genetic targeting and cell selectivity.

Jianxuan Zhang, Ph.D.

Weill Medical College of Cornell University

Development of a Protein-Knockout Library Screening System for Functional Interrogation of Tumorigenic Signaling Pathways

Jianxuan Zhang, Ph.D.¹, Xiujuan Chen¹, Xiaoling Lu¹, Liming Dong², and Pengbo Zhou¹

¹Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, NY, ²Pro Biomed LLC, Mountain View, CA

Protein knockout is a novel technology we developed, which harnesses the specificity of the SCF (SKP1, CUL1, F-box-containing substrate receptor, and RING domain protein Rbx1/Roc1/Hrt1) ubiquitination machinery to direct degradation of cellular proteins at will. We have extensively modified the protein knockout vectors and successively targeted a number of cellular proteins for destruction. The goal of the current study is to explore the application of the protein knockout system as a non-biased reverse genetic tool for functional identification and assignment of novel components in cellular signaling pathways. The rationale is that a lentiviral-based protein knockout library will be constructed for non-selective (random) targeting of cellular proteins for destruction, and clonal cells that are scored positive for the specific signaling pathway of interest will be selected and target protein identified that accounts for the altered phenotype. We have extensively optimized the protein knockout vectors and demonstrated selective destruction of a number of cellular proteins, including c-myc, b-catenin, Rb, p107, p130, BCL-6, Cdk2, and PCNA.

As a further proof-of-concept experiment, we showed recently that the engineered b-TrCP ubiquitin ligase could target not only soluble nuclear and cytoplasmic proteins, but also transmembrane receptor tyrosine kinases such as erbB-2 and EGFR that often initiate the activity of a given signaling pathway upon ligand engagement. Moreover, we have demonstrated unique properties for protein knockout technology that further enhance the potency and versatility of abrogating protein functions, which are not attainable by gene knockout, RNAi, antisense oligodeoxynucleotides, and ribozyme

technologies. These include simultaneous destruction of an entire protein family with redundant functions, selective targeting of a subpopulation of target protein that is either posttranslationally modified or resides in a specific subcellular compartment, and targeted eradication of proteins across different vertebrate species. We are currently constructing our first generation of protein knockout library to explore novel effectors and regulators of Fas-induced apoptosis pathway.

As an initial proof-of-efficacy experiment, we have constructed an engineered b-TrCP-DED ubiquitin ligase to target degradation of pro-caspase 8, a known mediator of Fas signaling, and is currently evaluating whether we can block Fas-induced cell death in Jurkat cells. We have also established lentiviral-based gene delivery vehicle and achieved efficient transduction in a variety of cell lines and primary hematopoietic cells. Successful completion of the proposed studies will provide a new and powerful proteomic tool for functional identification and assignment of cellular proteins involved in normal or tumorigenic signaling pathways, and for discovery and validation of potential therapeutic targets of cancer and other human diseases.

Yingming Zhao, Ph.D.

UT Southwestern Medical Center at Dallas

A Proteomics Approach to Studying Protein Farnesylation Using Tagging-via-Substrate Technology

Yingming Zhao, Ph.D.¹, Yoonjung Kho¹, Sung Chan Kim¹, Chen Jiang², Deb Barma¹, Sung Won Kwon¹, Jingke Cheng¹, Janis Jaunbergs¹, Fuyu Tamanoi², and John Falck¹

¹*Department of Biochemistry, UT Southwestern Medical Center at Dallas, Dallas, TX,*

²*Department of Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA*

Objectives

To develop an efficient method for detection and proteomics of farnesylated proteins.

Methods

A tagging-via-substrate (TAS) method involves metabolic incorporation of a synthetic azido-farnesyl analog and chemoselective derivatization of azido-farnesyl-modified proteins by the Staudinger reaction using a biotinylated phosphine capture reagent. The resulting protein conjugates can be specifically detected and/or affinity-purified by streptavidin-linked horseradish peroxidase or agarose beads. The isolated proteins are digested by trypsin; the resulting peptides are analyzed by HPLC/MS/MS for protein identification.

Results to Date

We synthesized the reagents for the experiments, including azide farnesyl diphosphate (the substrate for protein farnesylation) and biotinylated phosphine capture reagent (for conjugation to azide farnesylated proteins). Metabolic labeling of COS-1 cells with an

azido-containing substrate in the presence of lovastatin results in azido-farnesyl modification of Ras, restoration of its membrane association and Ras-dependent MEK activation. Reverse lovastatin-induced apoptosis was observed in H- ras -transformed NIH3T3 cells treated with azido-farnesyl substrates. These results suggest that azide farnesyl diphosphate could be used by the cells for farnesylation and azide-farnesylated proteins behave similarly to the naturally farnesylated proteins. The azido-farnesylated proteins were isolated for mass spectrometric analysis. Multiple farnesylated proteins were identified.

Plans for the Future

Applying the technology to profile protein farnesylation in multiple cancer cell lines with differential sensitivities to farnesyltransferase inhibitors (FTIs) in order to understand the mechanism of FTI functions.